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Dynamics of Histone H1 Exchange in Live Cells

By

Melody Anne Lever 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree Master of Science

In

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Dynamics of Histone H1 Exchange in Live Cells submitted by Melody Anne Lever in partial fulfillment of the requirements for the degree Master of Science in Medical Sciences – Oncology.

ABSTRACT

Histone H1 functions in the compaction of chromatin into higher-order structures. Modulation of H1 binding activity is thought to be an important step in the potentiation/depotentiation of chromatin structure for transcription. H1's ability to stabilize the folding of chromatin into a higher order structure enables it to act as a general repressor of transcription. Histone H1-GFP fusion proteins have been demonstrated to associate with chromatin in an apparently identical fashion to native histone H1. This provides a means to study histone H1-chromatin interactions in living cells. In this study, we have used human cells with a stably integrated human H1.1-GFP fusion protein to directly monitor histone H1 movement by fluorescence recovery after photobleaching (FRAP) in living cells. We find that exchange is rapid within both condensed and decondensed chromatin, occurs through a soluble intermediate, and is highly modulated by phosphorylation of its C-terminal tail.

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CHAPTER 1: INTRODUCTION

Section 1.1. Overview of Chromatin

It was recognized at the beginning of the twentieth century that the genome is packaged as discrete structures, called chromosomes. It was then observed at the completion of mitosis that chromosomes decondense into "interphase chromatin." Chromatin is comprised of DNA, histones and a number of nonhistone proteins and is responsible for the linear compaction of DNA that is necessary for it to fit into the small diameter of the nucleus. The basic repeating structural unit of chromatin, responsible for generating the beads in the well known "beads-on-a-string" chromatin conformation, is the nucleosome. The nucleosome consists of approximately 146 base pairs of DNA wrapped around a histone octamer core, consisting of two of each of the histones H3, H4, H2A and H2B[1,2]. In addition, an average of one histone H1 molecule binds to the periphery of each nucleosome (Figure I). This seals approximately 168 base pairs of DNA into two helical turns around the nucleosome, forming a structure called the chromatosome. Furthermore, binding of histone H1 helps drive and stabilize the folding of chromatin into higher-order structures[3,4]. Although a number of key factors have been identified as playing a role in modulating chromatin structure, there is little known about how they function. Investigation into the proteins or events that regulate chromatin remodelling is necessary if we are to better understand such cellular events as

transcriptional activation or gene silencing that occurs as a cell responds to external stimuli, progresses through the cell cycle, or undergoes transformation. The nucleosome and associated histones are the best defined players in regulating chromatin structure and dynamics. Post-translational modifications of both the core histones and histone H1, which introduce an enormous amount of potential heterogeneity in the nucleosome, have been demonstrated to play a dramatic role in chromatin remodelling[5-7]. Furthermore, co-ordinated binding of multiple nonhistone proteins, as well as the formation of multi-protein complexes, have been shown critical for regulating chromatin structure and thus altering gene expression[8-12].

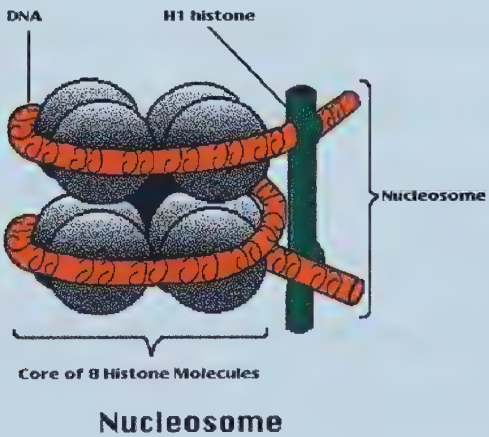

Figure I. *Structure of the nucleosome*. The basic repeating structural unit of chromatin is the nucleosome. The histone octamer core consists of two of each of the histones H3, H4, H2A and H2B. Approximately 146 base pairs of DNA wraps twice around the octamer core and is stabilized by histone H1. Adapted from Kornberg, 1999.

Section 1.2. Review of the Literature

1.2.1. Chromatin Organization

Chromatin has historically been divided into two categories, each of which has distinct characteristics. Euchromatin, is associated with transcriptionally active DNA, while heterochromatin generally contains DNA in a repressed state[13]. Heterochromatin was first defined cytologically over 70 years ago as that fraction of the genome that remains visibly condensed throughout the cell cycle[14]. More recently, heterochromatin has also been used to describe a number of functionally and structurally specialized forms of chromatin, including that associated with telomeres as well as centromeres. Variations of heterochromatin are seen in a number of widely divergent species and generally contain different regulatory proteins; however, there is no concrete theory that unites heterochromatic structures with their function. In spite of the differences between the specialized forms of heterochromatin, there are a number of similarities that classify them. The main characteristics of heterochromatin include: late S-phase replication, low gene density, an enrichment of specific nuclear proteins, contains large blocks of repetitive DNA, generally packaged into a structure that is relatively inaccessible to DNA-modifying reagents and can suppress the transcriptional activity of active genes translocated adjacent to it[15-17]. In contrast, euchromatin is relatively decondensed and contains a large amount of transcriptionally active DNA sequences[16,18-21]. Further investigation of the differences between

heterochromatin and euchromatin will undoubtedly lead to a better understanding of how and why chromatin structure is strictly regulated.

1.2.2. Nonhistone proteins and chromatin regulation

When placed next to heterochromatin, either by chromosomal rearrangement or trans-recruitment, euchromatic genes usually undergo a heritable cell-specific inactivation, leading to a mosaic or variegated pattern of gene expression[13,22-25]. In addition, with the exception of a few endogenous genes[16], genes artificially introduced or translocated into heterochromatin are subject to transcriptional silencing[16,26,27]. Variegation is thought to be caused by the ability of the condensed, higher-order heterochromatin to spread into neighbouring euchromatin and to cell-specifically silence transcription[13]. This "position effect variegation" (PEV) phenomenon is believed to be driven by nonhistone heterochromatin binding proteins. These proteins were first isolated in *Drosophila* as mutations that can either increase or reduce the proportion of cells in which inactivation occurs. More than 50 candidate heterochromatin proteins have been reported in *Drosophila* alone[28,29]. Two of the most well defined nonhistone proteins in higher eukaryotes are heterochromatin binding protein 1 (HP1) and chromatin assembly factor-1 (CAF-1.), each of which has been shown to associate with nucleosomal histones.

HP1 was first identified in a screen for monoclonal antibodies that stained *Drosophila* polytene chromosomes[30,31]. The gene Su(var)2-5 has since been

shown to encode for HP1[32-35]. This gene suppressed PEV when deleted and enhanced PEV when duplicated, indicating that HP1 is an essential component of heterochromatin and is required in a precise stoichiometry to properly set and/or maintain the inactivated state of genes that are subject to PEV[32,33,36]. HP1 orthologs have been identified in yeast, nematode, chicken, frog, and mammals[37-40]. Three distinct mammalian HP1s, HP1α, HP1β and HP1γ have been characterized and exhibit differential localization with, however, some accumulation at pericentromeric heterochromatin[41-45]. The majority of HP1α and HP1β are localized on centromeres, while HP1γ is found on discrete regions on chromosome arms[46,47]. Both HP1α and HP1γ are phosphorylated during interphase and throughout the cell cycle, although they are more highly phosphorylated during mitosis[47]. These HP1s have been shown to silence transcription when directly tethered to DNA[44,48]. These findings support the hypothesis that binding of HP1s to chromatin could play a role in gene silencing. Both mouse and human HP1s have also been found to directly interact with the transcriptional corepressor TIF1β *in vivo*[44,48]. Nielsen et al., suggested that the HP1-TIF1β complex might silence transcription of specific genes by inducing the formation of, and/or juxtaposition with, heterochromatin[44]. However, the details of the molecular mechanism involved remain unclear. Recently, *Drosophila* HP1 (dHP1) has been shown to bind to nucleosomes and DNA *in vitro*, indicating that these interactions could be relevant to HP1-mediated heterochromatin assembly and silencing[49].

Based on amino acid sequence similarities, a typical domain structure has been ascribed to members of the HP1 family, comprising an N-terminal *chr*omatin *o*rganization *mo*difier, or chromodomain (CD), and a structurally related C-terminal chromoshadow domain (CSD) connected by a poorly conserved hinge region[50]. In contrast to the CSD, the CD is not specific to the HP1 protein family; it has been found in a wide variety of proteins, many of which function in chromatin organization and gene regulation[37,51]. The approximately 50 amino acid N-terminal chromodomain shares homology with polycomb, an important regulatory gene that functions in the stable repression of homeotic genes during *Drosophila* development[52]. The chromoshadow domain, located near the HP1 C-terminus, appears to be a key element in the assembly of multiprotein complexes in heterochromatin[37]. Emerging evidence suggest that HP1 is a structural adapter whose role is to assemble macromolecular complexes to silence chromatin. In support of this, HP1 has been shown to interact with a number of nuclear proteins associated with DNA binding, direct and indirect interactions with components of replication machinery, the nuclear lamina, and various other nonhistone chromosomal proteins. Such proteins include itself[43,48]; the lamin B receptor of the inner nuclear envelope[43,48]; BRG1/SNF2b, a component of the SWI/SNF complex[43,48]; nuclear receptor cofactor TIF1-α;[43,48] bLAP, a leucine amino peptidase[43,48]; a RAD54-like presumed helicase[43,48]; the product of the Su(var)3-7 gene, a heterochromatin protein of unknown function[53]; and SP100, a major component of PML nuclear bodies[54]. These interactions are believed to occur

through their CSDs. Furthermore, the localization of HP1 to pericentric heterochromatin has been found to be dependent upon binding to the origin recognition complex (ORC).[55] Although ORC was originally identified as a protein involved in initiation of DNA replication, it was subsequently found to play an independent role in heterochromatin structure and function in both *S. cerevisiae* and *Drosophila*[55,56]. The protein(s) that anchor HP1 to telomeres, if any, are not known. Interestingly, loss-of-function mutations in the HP1 gene also result in a number of structural defects in mitotic chromosomes including a lack of chromatin condensation and telomere fusions that often generate chromosome breaks[44]. In addition, mutations in *S.pombe* Swi6p, which is structurally similar to HP1, results in a high rate of chromosome loss and an increased incidence of lagging chromosomes during anaphase[57], demonstrating the permissive role of these heterochromatin binding proteins. Another well-known heterochromatin remodelling protein found in higher eukaryotes was identified as the chromatin assembly factor-1 (CAF-1).

CAF-1 comprises three polypeptide subunits: p150, p60 and p48 (cac1p, cac2p and cac3p in *S. cerevisiae*). During DNA replication, CAF-1 deposits newly synthesised and acetylated core histones H3 and H4 into nascent chromatin[58,59]. At least in humans, CAF-1 is found to be a general nucleosome assembly factor that is targeted to both euchromatic and heterochromatic DNA replication foci[60]. Deletion of the genes encoding any of the three *S. cerevisiae* subunits of CAF-1 (cac1p, cac2p or cac3p) results in silencing defects of reporter genes packaged into telomeric heterochromatin[61,62]. Interestingly, recent studies have shown that, through its

ability to bind HP1 proteins, the N-terminal domain of CAF-1 p150 plays a role in the maintenance of heterochromatin that is distinct from the role of p150 in nucleosome assembly during S phase[63]. Interaction with this subunit may be necessary to indirectly stabilize the binding of HP1 proteins to heterochromatin by binding to histones, or it may be transiently required during heterochromatin replication, in order to re-establish heterochromatin structure after its been disrupted during replication. However, details of this remain unclear and further study is needed to better understand the function of the interactions between CAF-1, HP1 and other chromatin binding proteins, including histones.

1.2.3. Histone modifications and chromatin remodelling

Modification of the core histones has been demonstrated to play a major role in chromatin remodelling[5,6,64,65]. The histone octamer core is arranged as a $(H3\text{-}H4)_2$ tetramer with two H2A-H2B dimers positioned on either side[2]. In contrast, histone H1 binds to the linker DNA, which joins adjacent nucleosomes, and to the core histones, H2A and H4, at a ratio of approximately one histone H1 molecule per nucleosome[2]. The core histones are evolutionarily highly conserved, small globular proteins. They have a similar structure with a hydrophilic N-terminal unstructured domain that can protrude from the nucleosome core particle, a globular domain organized by the histone fold and a C-terminal unstructured tail. In addition, they are susceptible to a wide range of posttranslational modifications including acetylation, phosphorylation, methylation, ubiquitination, glycosylation and ADP-

ribosylation. Most modifications occur on the basic N-terminal tail domain and are involved in a number of chromatin structural states[1]. Because acetylation and phosphorylation occur at specific positions in the histone N-termini, they have been proposed to impart a 'histone code" for the dynamic restructuring of chromatin and the transmission of epigenetic information[66]. Histone modifications could allow both transient and more long-term alterations in chromatin structure, so that the cell can adjust gene activity to changes in growth and differentiation conditions, but also maintain specific gene expression programmes in order to inherit cell-type identities. Nuclear enzymes regulating the turnover of acetyl- and phosphoryl-groups on histone N-termini have been isolated[67-69], confirming that these modifications largely present short-term signals of the 'histone code'. In contrast, histone methylation has been regarded as a more long-term epigenetic mark, consistent with the relatively low turnover of the methyl group. Recent findings in this field have led to a more detailed understanding of the role(s) of histone methylation. The first lysine histone methyltransferase (HMTase) and its associated functions, was identified by Rea et al[70]. Interestingly, it is a mammalian homolog of a gene product encoded by the dominant *Drosophila* suppressor of "position effect variegation" (PEV), Su(var)3-9[71]. Remarkably, this enzyme selectively methylates the core histone H3 at Lysine 9[70]. Because of these intriguing genetic and biochemical parallels, this HMTase was named SUV39H1. Furthermore, it is known that SUV39H1 and HP1 are complexed together[72-74]. Therefore, the SUV39H1 HMTase can propagate an epigenetic signal that is then possibly stabilized by interaction with HP1 proteins. Interestingly, SUV39H1 has been shown to almost

completely dissociate from centromeric positions at anaphase, whereas a significant fraction of HP1 remains chromosome bound throughout mitosis.

Another posttranslational modification associated with mitosis is the hyperphosphorylation of histone H3. It was demonstrated that the condensation of mitotic chromosomes correlates with the increased phosphorylation of H3[75]. Furthermore, it has recently been shown that inactivation and dissociation of CAF-1 p150 from heterochromatin occurs in parallel with modification of histone H3 as cells prepare for mitosis[63]. Both phosphorylation of H3 and dissociation of CAF-1 occur well before the visible onset of mitosis. In addition, rebinding of MOD1 (one of the mouse HP1 family members) to mitotic chromosomes also parallels H3 dephosphorylation[63], demonstrating the tight relationship between these nonhistone heterochromatin proteins and the nucleosomal histones throughout the cell cycle. In addition, all three mammalian HP1s are known to specifically interact with the histone-fold of histone H3[50,76]. Furthermore, HP1α interacts with histone H1[76]. Like HP1α, *Drosophila* HP1 interacts with both histones H1 and H3[76], indicating that these interactions are conserved across species. Nielson et al., further illustrate that HP1α contains two specific histone binding domains; the N-terminal CD, contains an interaction domain for H3; whereas the less conserved hinge region is sufficient for interaction with histone H1. They also show that the polycomb CD similarly interacts with histone H3. Thus, this activity is likely to be shared by other, if not all, members of the chromo superfamily. Many researchers suggest that the interactions between some nonhistone heterochromatin proteins and specific histone

modifications correlated with mitosis, results in dissociation of a number of heterochromatin binding proteins and allows access to various proteins required to promote faithful mitotic chromosome segregation. Although this explanation is intriguing, it is obvious that further investigation is needed to fully understand the function of the dynamic changes in heterochromatin that result from regulated binding and modification of both nonhistone and histone proteins. We have chosen to begin addressing these questions by characterizing the binding dynamics of the nucleosomal histone, H1.

Section 1.3. Histone H1

1.3.1. Structure, binding and function of histone H1

Histone H1 is thought to primarily bind nucleosomal DNA at the pseudodyad and at the linker DNA (which joins adjacent nucleosomes) as it enters and leaves each nucleosome core particle[77,78]. For this reason, histone H1 is commonly referred to as linker histone. Histone H1 has a tripartite structure consisting of a central globular region, rich in hydrophobic amino acids, and basic C- and N-terminal tails[79]. These terminal domains contain sites of phosphorylation and most likely interact with LDNA of nearby nucleosomes to stabilize chromatin, possibly by neutralizing charges on DNA[80]. The binding of the central globular domain, GH1, is more of a mystery. Previous nuclear digestion experiments suggest that it is bound to the outward-facing minor groove of DNA at the center of the nucleosome, where

the dyad axis of symmetry passes through the DNA[81,82]. This model has also been supported by DNase I footprinting[83]. However, comparison of the globular domain structure with other DNA binding proteins implicates its association with the major groove of DNA[84,85]. Electron microscopy has revealed that, when histone H1 is present, the linker DNA enters and exits the nucleosome from a single region; however, removal of histone H1 shows that the sites of DNA entry and exit become plainly distinct[86-88]. These and other observations led to the view that histone H1 is located over the nucleosomal dyad-symmetry axis, binding simultaneously to a pair of DNA segments entering and leaving the nucleosome.

Histone H1 variants are found in almost all species ranging from bacteria to humans. In non-mammalian species one to eight variants have been shown to be expressed, while there are seven variants in mammals; 4 somatic and 3 differentiation-specific. A number of different nomenclatures are frequently used to describe the histone H1 variants. For our work, unless otherwise cited, we are referring to Doericke's nomenclature. In humans, the somatic variants are referred to as histones H1.1-H1.4 and the differentiation-specific are termed H1a, H1t and $H1^0$. These proteins differ in their binding to mononucleosomes and in their abilities to aggregate chromatin[89-91]. Immunostaining with antibodies revealed that a subset of histone H1 subtypes are distributed non-uniformly throughout the nucleus, with histones H1.2 and H1.4 showing punctate patterns and histone H1.3 staining the periphery of the nucleus[89-91]. In contrast, histone H1.1 has a more uniform staining pattern, correlating with the intensity of the DNA staining. Throughout evolution, there has been large

variation in both structure and function of histone H1. However, the highest degree of conservation is seen in the central globular domain (integral for DNA binding) while the most drastic variation is seen in both the N- and C-terminal tails (sites of posttranslational modifications[92,93]; Table I). Comparison of the amino acid sequences revealed that histone H1.1 showed more divergence in structure from the other subtypes[94]. Such compartmentalization suggests that each of the histone H1 subtypes may play distinct roles in organizing chromatin for different functions. This was further supported by studies of the mouse histone, H1a, showing that this subtype colocalized with transcriptionally active regions, and that phosphorylation of this subtype could function to maintain the chromatin in a transcriptionally competent and decondensed state[95,96]. Specific functions for histone H1 subtypes were further demonstrated when it was reported that the overexpression of histone H1c (similar to human H1.3) in mouse cells increased the transcript levels of some genes, while overexpression of histone $H1^0$ led to an overall decrease in transcript levels[97]. In addition, in a chicken B cell line that has 6 subtypes of histone H1, deletion of histone clusters to eliminate 5 of the subtypes did not affect the growth rate of the cell, nor was the global chromatin structure altered[98]. Only a single copy of histone H1 was sufficient for cell proliferation, but two-dimensional gel electrophoresis showed that overall protein expression was affected. This result suggests some redundancy in histone H1 function, although it is not known if this is true of mammalian cells.

Table I. *Similarity of H1 cDNAs*

	Full-length cDNAs				Globular domains				C-terminal domains			
	H1.2	H1.3	H1.4	H1a	H1.2	H1.3	H1.4	H1a	H1.2	H1.3	H1.4	H1a
H1.1	63	59	63	68	73	77	69	71	43	52	46	53
H1.2		62	58	70		83	75	79		63	51	62
H1.3			57	53			73	77			54	52
H1.4				67				81				51

The percentage of identical nucleotides was calculated from a multiple alignment of the cDNAs. The initial alignment was generated with the program ClustalW (adapted from Eick et al., 1989).

Although histone H1 is one of the most abundant proteins in the nucleus and has been studied extensively, answers to the most basic questions concerning histone H1, such as its location and function, remain controversial or unanswered. Until the mid 1990's, the majority of studies on histone H1 binding had been limited to *in vitro* analysis. Major advances to understanding chromatin assembly processes came from *in vitro* studies of Xenopus eggs. These cells contain large stores of histones, which provide a source of material for chromatin assembly. Although these and other studies using purified components have increased our knowledge of chromatin structure, including histone dynamics, there are a number of disadvantages.

One major limitation is the time required for assembly of nucleosomal arrays *in vitro*. In Xenopus egg extracts chromatin assembly on double stranded DNA requires over one hour to generate a nucleosomal array and does not require synthetic processes such as DNA replication or protein synthesis[99,100]. It has been

well established that chromatin assembly *in vitro* is much less efficient than *in vivo* assembly. This suggests that other factors, not available *in vitro*, are being used for chromatin remodeling in living systems. In addition, the physiological spacing of nucleosomes found in the chromosome is not seen *in vitro*. Histone octamers reconstituted by dialysis from high salt concentrations pack together on DNA as closely as possible and are found every 150-160 base pairs (bps) apart as there is no linker DNA[99]. Normally, nucleosomes are spaced approximately 170-180 bps apart[2]. This close packing is to maximize not only strong protein-DNA interactions, but also protein-protein interactions along the DNA backbone. Furthermore, appropriate histone deposition is not seen *in vitro* as it is *in vivo*[101,102]. Following dialysis from high salt an entire unmodified, irregular histone octamer is deposited onto DNA[102]. Consequently, histone H1 interacts with long exposed stretches of free DNA forming aggregates and causing DNA to precipitate. In fact, two populations of chromatin become apparent in using *in vitro* systems. One fraction is termed aggregation-prone (precipitated DNA and proteins) and the other is referred to as aggregation-resistant[102]. This phenomenon may help explain differences between our studies of histone H1 dynamics and *in vitro* experiments reported by such groups as Caron and Thomas or Jin and Cole as discussed in chapter1.3.4.

Although there is still debate as to the exact binding location of histone H1, it is known to stabilize the folding of 30nm chromatin fibres and most likely higher order forms of chromatin[82,87,88,103]. It can be released from chromatin by increasing the salt concentration, leaving nucleosomes otherwise intact[104]. This enables us to

further study its location and function. When histone H1 is not present, chromatin folds into irregular clumps, rather than into the more highly ordered 30nm fibres, which are characteristic of native chromatin[88,104]. Higher than normal salt concentrations are required for any folding that can occur at this point. These observations imply that histone H1 contributes to the free energy of chromatin folding. This also suggests that binding of histone H1 helps select a single folded state from among the set of compact states that may be reached in its absence. Modulation of histone H1 binding activity is thought to be an important step in the potentiation/depotentiation of chromatin structure for transcription[3,4,105,106].

1.3.2. Histone H1 and transcription

Although controversy surrounds the mechanisms involved, histone H1 has been shown to act as both a specific and general repressor of transcription[3,4,105,106]. Its ability to stabilize folding of chromatin into higher order structures enables it to repress transcription. Its role in this repression has been demonstrated through a number of mechanisms. Possible modes of action are: (1) to sterically inhibit the binding of necessary transcriptional machinery[107,108]; (2) to alter nucleosome positioning and thus reposition factor binding sites; and finally (3) as an indirect pathway for histone H1 transcriptional repression[108]. Evidence for the first example comes from studies of histone H1 and core histone acetylation. Herrera et al., have shown that the linker histones H1 and H5, specifically inhibit the acetylation of mono- and oligonucleosomes and not that of free histones of histone-DNA

mixtures[109]. This repression is due to steric hindrance of histone H3 by the H1 histone tails and not due to condensation of the chromatin fiber. It is well known that there is a strong correlation between the acetylation state of core histones, the degree of chromatin compaction and the transcriptional competence of specific genes[65,110,111]. Indeed, a clear repressive role for chromatin structure has been established by detailed biochemical studies[112,113]. They reveal that the activities of RNA Pol II and III are significantly inhibited by assembly of target sequences into nucleosomes[114,115]. In support of the second model, it was demonstrated that histone H1 is capable of reducing nucleosome mobility[116] and locking the nucleosomes into a unique position concurrent with inhibition of transcription, is likely due to inaccessibility of transcription factor binding sequences. Furthermore, global repression was seen in studies on *Xenopus*. At the mid-blastula transition, the appearance of histone H1 caused the repression of the 20,000 oocyte 5S genes (per haploid number), but did not affect the continued expression of the 400 somatic 5S genes[117]. Moreover, Wolffe demonstrated that the highly conserved globular domain alone of histone H1 is sufficient to cause this switch in 5S gene expression. It has been extensively shown in *Xenopus borealis* and *X. laevis* 5S chromatin that the differential positioning of histone octamers (stablized by histone H1) on the oocyte and somatic genes explains the selective repression of the oocyte genes (reviewed in)[4]. The coding sequences of the oocyte and somatic genes are virtually identical, but the flanking sequences are, respectively AT- or GC-rich. The differential positioning of the core histones results in different degrees of occlusion of the binding sites for TFIIIA and hence in different relative affinities of the oocyte

and somatic sequences for TFIIIA and histone H1[118]. A nucleosome on the oocyte sequence has a higher affinity for the histone than for TFIIIA, whose binding site is largely buried within the nucleosome, and is therefore repressed[119]. The somatic 5S nucleosome binds TFIIIA (whose binding site is relatively exposed at the periphery of the nucleosome or in the linker DNA) in preference to histone H1 and remains active. The consequences of these effects are reinforced by the increase in histone H1 and decrease in TFIIIA concentration after the mid-blastula transition. An investigation of *Xenopus laevis* somatic and oocyte 5S genes also found differential positioning and proposed selective formation of a repressive histone H1-containing chromatin structure on the oocyte genes[119]. It was illustrated that direct binding of the histone does not inhibit transcription of *X. laevis* somatic 5S nucleosomes[120,121]. The consequences of occlusion of the TFIIIA binding site might well assemble into an histone H1-dependent, repressive, higher-order structure; nonetheless, the root cause of the selective repression of the oocyte genes appears to be nucleosome positioning to occlude a transcription factor binding site and permit histone H1 binding. Also relevant, is the observation that the upstream regions of active *X. laevis* somatic 5S rRNA genes, but not oocyte genes, are hyperacetylated *in vivo*, which probably maintains the somatic 5S chromatin in an unfolded state[121].

In addition to the more global repression of oocyte genes in *Xenopus*, Juan et al., demonstrated that histone H1 specifically inhibits binding of the basic helix-loop-helix upstream stimulatory factor (USF)[122]. In these studies, they show that unlike

TFIIIA binding, histone H1 repression of USF binding was independent of nucleosome mobility, indicating an alternate source of histone H1 repression. Instead, their data is consistent with a third mechanism by which this linker histone stabilizes the core histone octamer-DNA interactions, thus indirectly reducing transient exposure of factor binding sites. By using reconstituted nucleosome cores, they have shown that the histone octamer takes up a precise location on particular DNA fragments of 183-bp; from 10 to 156 base pairs relative to the 5'-end. This position was unchanged after binding of either histone H1 or USF[122]. Thus, histone H1-mediated repression of USF binding in this instance involved neither changes in nucleosome position nor a reduction of nucleosome mobility. This example, therefore, clearly illustrates a mechanism of histone H1 repression, which is independent of movement of the histone octamer. In these studies, the researchers have made use of the fact that there are two USF binding sites with differential histone H1 repression to further investigate this indirect pathway of repression.

The USF binding site, 35 bp into the nucleosome core is more highly repressed than the binding site that is 9 bp into the core. Interestingly, if histone H1 binds asymmetrically to reconstituted nucleosome cores (not across the DNA pseudodyad of the nucleosome), as proposed by Pruss and colleagues (see chapter 1.3.), then the less repressed USF binding site 9 bp into the nucleosome core would be located directly at the proposed site of histone H1 binding whereas the highly repressed site 35 bp inside would be well beyond the proposed histone binding site. Polach and Widom have proposed a dynamic equilibrium model for transcription factor access

to recognition elements within nucleosomes, which is consistent with several *in vitro* experiments[123,124]. They suggest that DNA on the surface of the histone octamer is dynamic, transiently exposing stretches of DNA to transcription factors. Importantly, site exposure decreases as the site is moved further into the nucleosome core, consistent with an "unpeeling" mechanism and numerous reports of translation "position effect". Thus, sites deeper into the nucleosome core are more sensitive to the extent of DNA unpeeling. Histone H1 has been shown by physical studies to stabilize the interaction of linker DNA and additional DNA within the nucleosome core with the histone octamer[125-127]. Moreover, microscopic studies on topological analysis further support a role of histone H1 in preventing the "peeling" of DNA from the surface of the histone octamer[105,128]. The observation by Juan et al., that histone H1 represses factor binding to a site 35 bp into the nucleosome core[122] (where histone H1 is not thought to interact directly) is consistent with the model whereby the histone indirectly represses factor binding by reducing transient unpeeling of DNA from the histone octamer surface, thus "sealing" the nucleosomal DNA. By this mechanism, histone H1 could lead to repression of factor access over a broader region of DNA beyond its specific sites of direct interaction. These results are also consistent with protein-DNA interaction experiments demonstrating that cross-linking of histone H1 to actively transcribed genes is significantly lower than to transcriptionaly silent genes[83,129-132].

In addition to its repressive effects, histone H1 has been shown to act as a specific activator of transcription. In *Tetrahymena*, deletion of this histone led to specific

gene activation without a pronounced effect on global transcription by Pol II or Pol III[106]. Furthermore, studies by Archer and Lee on the mouse mammary tumour virus (MMTV) suggest that, once phosphorylated, histone H1 enables activation of genes under control of the MMTV promoter stably transfected into mammalian cells[133]. Their experiments also demonstrate that the phosphorylation state of histone H1 parallels the activation status of the promoter. They find that when histone H1 phosphorylation is blocked, the MMTV promoter will be refractory to activation. This suggests that phosphorylation acts to weaken the interaction between the histone and DNA and thus allow local remodelling of the chromatin that is necessary for gene activation in that region. Interestingly, they also show in contrast, that both the endogenous metallothionein promoter and a transiently transfected MMTV promoter into the same cells are not influenced by the phosphorylation status of histone H1[133], demonstrating that this is not a global transcriptional event that may be due to overall chromatin remodelling. This suggests that a specific chromatin conformation of the stably transfected MMTV promoter, necessary for normal activation, is regulated by histone H1 binding and phosphorylation and provides strong evidence that nucleosomal positioning and sliding may be the method by which histone H1 binding is selectively activating or inhibiting specific genes.

1.3.3. Phosphorylation and histone H1

The primary posttranslational modification of histone H1 is phosphorylation. This phosphorylation appears to be cell cycle-dependent as the histone is transiently phosphorylated throughout the cell cycle, beginning initially after it is synthesized. It is minimally phosphorylated during early G1; however, like histone H3 it becomes maximally phosphorylated during mitosis[134]. Variants of mammalian histone H1 contain up to five possible sites of phosphorylation, the majority of which are on the C-terminal tail[135]. It has long been thought that phosphorylation, by cyclin-dependent kinases, weakens its association with chromatin due to charge repulsion of the DNA phosphate backbone. This loosening results in an unravelling of the condensed region allowing proteins, such as transcription factors, to enter and bind. However, little is known about the role of specific phosphorylation sites in histone H1 binding. Some researchers suggest that during mitosis, when histone H1 becomes hyperphosphorylated, specific phosphorylations correlate with the further folding of chromatin[135]. Gorovsky and Dou made an instrumental finding in 1999 studying two very specific strains of *Tetrahymena*[100]. They generated one strain in which all histone H1 phosphorylation sites were mutated to alanine, thus preventing phosphorylation. The other strain had the same sites changed to glutamic acid, mimicking the charge of the fully phosphorylated state. Remarkably, the mutation that mimics histone H1 phosphorylation phenocopies histone H1 removal with regard to the basal activation of one reporter gene (ngoA) and repression of the induced expression of another (CyP1)[100]. In the absence of histone phosphorylation, the time required for induction of CyP1 expression is drastically reduced and in wild-type cells, CyP1 expression only occurs after histone H1 is

dephosphorylated[100]. This data strongly argues that phosphorylation can regulate transcription and mimics the loss of histone H1 from chromatin. Moreover, the mutant strains they used were constructed such that they would mimic the charge of the phosphorylated region without mimicking the structure or increased hydrophilicity of the phosphorylated amino acids. Therefore, these results also demonstrate that phosphorylation of histone H1 may not act through phosphate recognition or creation of a site-specific charge to alter its binding, but rather that phosphorylation changes the overall charge of a small domain. Although these experiments provide strong evidence that phosphorylation weakens the binding of histone H1 to chromatin, they do not give insight into the role it plays in the dynamics of its exchange, which has led to the latter half of our experiments.

1.3.4. Dynamics of histone H1 exchange

In vitro studies by Jean Thomas and colleagues in the early 1980's pioneered the investigation into the dynamics of histone H1 exchange. They showed that at low ionic strength there was little to no exchange of the histone between isolated chromatin fragments; however, as the NaCl concentration was increased to physiological ionic strength, there was complete exchange from one chromatin fragment to another[136]. Louters and Chalkley also demonstrated that increasing ionic strength acted to increase the maximum percent of exchange by approximately 40%[137]. They further stated that complete exchange of radiolabeled histone H1 occurred within two hours in isolated and intact nuclei[138], significantly slower than

what we have measured in living cells (Section 3.3.). From these studies, Thomas and others suggested that increasing ionic strength acts to neutralize charges on both DNA and histone H1 and thus weaken the interaction between the two[136,139]. They implied that histone H1 is then released into the nucleoplasm and exchanges as a freely mobile population. Jin and Cole extensively challenged this argument. Their work showed that histone H1 mobility was nonuniformly distributed within the nucleus and that exchange was restricted to particular classes of chromatin, independent of ionic strength[140]. They saw exchange within both the aggregation-prone and aggregation-resistant classes of chromatin that result from *in vitro* isolation (chapter 1.3.1.), but not between the two populations, as was seen by Caron and Thomas. Jin and Cole consistently showed that exchange was restricted to chromatin populations of similar composition[141]. Because the two *in vitro* chromatin populations have distinct biochemical properties that inhibit cross interactions, the researchers implied that this spatial separation is what restricted the exchange of histone H1 between the soluble and precipitated chromatin populations. They suggested that exchange is not dependent upon ionic strength but rather on fiber-fiber interactions and that histone H1 can only exchange directly from one chromatin fiber to another, without the transient release into solution. Interestingly, it was later demonstrated that increasing ionic strength acts to increase the number of chromatin fiber-fiber interactions[142]. This may support both hypotheses in that the increased ionic strength may indirectly increase histone H1 exchange by allowing more fiber-fiber interactions and thus enabling the histone to more frequently "jump" from one binding site to another. Furthermore, other studies show that in isolated

nuclei, histone H1 within the nucleus does not exchange with chromatin outside of the nuclear membrane, again indicating that direct fiber-fiber interaction may be required for exchange[143]. Together, this work suggests that histone H1 does not exchange through the nucleoplasm as a freely mobile population *in vivo*. In addition, because these experiments show complete exchange of histone H1 within fractionated chromatin, it has been well accepted that histone H1 dynamically interacts with chromatin and that its displacement does not require energy. Therefore, regardless of ionic strength or aggregation properties, if histone H1 were freely diffusible as seen *in vitro*, one would expect that cell viability is not a factor in the extent and rate of exchange. Thus, *in vivo*, the energy or phosphorylation status of the cell would not be a factor in histone H1's ability to exchange.

Section 1.4. Statement of hypothesis

There is relatively little known about the binding dynamics of histone H1 in living cells. In 1999, Gunjan et al., published the first data using green fluorescent protein (GFP)-tagged histone H1 variants[144]. They show that overexpression of the GFP-tagged histones does not lead to the formation of aberrant chromatin structures nor does it affect the intrinsic binding properties of histone H1 and can thus be used in a living system to monitor this histone. We have therefore used human H1-GFP fusion proteins with time-lapse live cell laser microscopy to monitor the distribution, rate and extent of histone H1 exchange as well as factors regulating this exchange in living human cells. Based on what is known about histone H1 thus far, we propose

that: *histone H1 is relatively stably bound during interphase and is modulated by its phosphorylation.*

Chapter 2: Materials and Methods

Section 2.1. Reagents, DNA Constructs and Antibodies

Reagents for cell culture, including Geneticin (G418), were purchased from GIBCO (Burlington, On.). Staurosporine was purchased from CalBiochem (Hornby, On.). 5,6 dichlorobenzimidazole ribozide (DRB), Sodium Azide, Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), 4-(2-Aminoethyl) Benzenesulfonyl Fluoride Hydrochloride (AEBSF) and 4',6-Diamidino-2-phenylindole Dihydrochloride:Hydrate (DAPI) were purchased from Sigma (Oakville, On.). Hoechst 33342 was purchased from Molecular Probes (Cedarlane; Hornby, On.). Micrococcal Nuclease was purchased from USB Corporation (Cleveland,Oh). 2-deoxyglucose was a gift of Drs. Laurina Tourcotte and Joan Turner (Edmonton, Ab.).

The red-shifted variant of wild type GFP, pEGFP-N2 (Clonetech) was used for the empty vector GFP cell line. pEGFP-N2 encodes the GFPmut1 variant, which contains the double amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. Polyadenylation signals from the Herpes Simplex Virus thymidine kinase (HSV TK) gene allows stably transfected eukaryotic cells to be selected using G418. PCR amplification was used to generate the Histone H1.1 and H1.2 (sequences published by Eick et al (1989)). HeLa genomic DNA was used as the template. Amplified H1.1 and H1.2 DNA was ligated into pEGFP-N1 (Clonetech) which was transformed

and grown up for the clone. All histone-GFP plasmids were a designed and constructed by Dr. John Th'ng (Ontario Cancer Care) as part of an ongoing collaboration.

Polyclonal antibody against total histone H1 was the gift of Dr. M. Parseghian (Irvine, CA). Polyclonal antibody against the phosphorylated species of histone H1 was a gift from Dr. D. Allis (Charlottesville, VA).

Section 2.2. Cell Culture

SK-N-SH stabley transfected cell lines: Human neuroblastoma (SK-N-SH) cells were cultured at 37°C, 5% CO_2, in Gibco culture media Dulbecco's modified Earle's Medium (DMEM) containing 10% Fetal Calf Serum (FCS). The six different H1.1-GFP variants/mutants as well as the two H1.2-GFP plasmids were each stabley transfected into SK-N-SH cells using LipofectAMINE Reagent (Gibco BRL Cat. No. 18324-012) and following the GibcoBRL protocol for stable transfection of adherent cells. 48 hours after transfection, media was supplemented with G418 at a concentration of 800ug/ml. Non-transfected cells were killed off within 10 to 14 days after addition of G418. Stable cell lines were maintained in 400 ug/ml G418.

A549 stabley transfected cell lines: Lung diploid fibroblast cells (A549-ATTC CTL-183) were cultured at 37°C, 5% CO_2, in Gibco culture media DMEM:F12 containing

10% FCS. Stabley transfected cell lines of each of the six H1.1-GFP and two H1.2-GFP constructs were generated using Effectine Reagent (Qiagen Cat. No. 301425) and following the Qiagen Effectine Transfection Reagent protocol for stable transfection of adherent cells. 24 hours after transfection, media was supplemented with G418 at a concentration of 200 ug/ml. Non-transfected cells were killed off in three stages over 14 days. Stable cell lines were maintained in 200 ug/ml G418.

8226 human multiple myeloma and Raji B cell lines stabley transfected with full length H1.1-GFP (8226-H1.1GFP and Raji-H1.1GFP, respectively) were kindly donated by Christopher Maxwell (CCI, Ab.) and cultured at 37^0C, 5% CO_2 in Gibco culture media RPMI 1640 containing 10% FCS and 0.025% G418.

Section 2.3. Analysis of the effects of GFP on Histone H1 Binding

2.3.1. Nuclei and Chromatin Preparation

Approximately 100 mls of each: untransfected SK-N-SH, H1.1-GFP transfected SK-N-SH (SK-N-H1.1GFP), 8226-H1.1GFP, Raji-H1.1GFP cells were cultured until 90% confluent/concentrated. Adherent cells were trypsonized for 10 minutes at 37^oC. Cells were collected by centrifugation at 1400rpm for 7 minutes at 4^oC, washed in PBS and centrifuged as before. Nuclei were isolated by lysing cells in RSB pH 7.5 (10 mM Tris-Cl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM sodium butyrate. Cells were centrifuged at 5000 rpm for 10 minutes at 4^oC. This step was

repeated twice. 1 mM AEBSF (Sigma Prod. no. A8456) was added at each step to minimize protein degradation. After isolation of nuclei, they were resuspended in a small volume of nuclei lysis buffer (Buffer B) and incubated at 37°C for 10 minutes. Micrococcal Nuclease was added for a further 10 minutes at a concentration of 40 units/ml. The lysis reaction was then stopped by adding EDTA to a final concentration of 10 mM and placed on ice for 30 minutes. The fractionated nuclei were then centrifuged at 10, 000 rpm for 10 minutes at 4°C to collect cell debris. The chromatin containing supernatant was dialyzed overnight at 4°C in 0.01 M Sodium Phosphate Buffer pH 6.8. Chromatin was frozen at –80°C, liophilized and stored at –20°C.

2.3.2. Hydroxylapatite Columns and Western Blot analysis

Hydroxylapatite Columns

The hydroxylapatite columns (BIO-RAD Econo-Pac CHT-II Cartridge, Cat. No. 732-0083) were prepared by washing with 0.01 M Sodium Phosphate Buffer pH 6.8 for 3 minutes at a flow rate of 0.8 ml/min, then 0.5 M Sodium Phosphate Buffer pH 6.8 for 10 minutes. The columns were left for 15 minutes to equilibrate. In order to minimize air bubbles, the columns were placed upside down until needed. Immediately prior to sample loading, the column was activated with 5-10 mls of 10 mM Sodium Phosphate buffer, pH 7.2 at a flow rate of 0.6-0.8 ml/min. The chromatin from each cell line was resuspended in 1mM Sodium Phosphate Buffer pH 6.8 and applied to individual columns after being filtered through a 0.45 um

filter. Each sample was run through the column twice and eluted with 6 mls each of elution buffers ranging from 150 mM NaCl to 600 mM NaCl in 0.01 M Sodium Phosphate buffer, pH 6.8 at a rate of 0.6 mls/minute. 1.5 ml fractions were collected and dialyzed overnight in dH_20 at 4°C with one change of dH_20 after 5 hours. Samples were then frozen at -80°C, liophilized, resuspended in SDS-PAGE sample buffer and run on a 15% SDS polyacrylamide gel to identify histone H1-containing fractions.

Whole cell protein lysates were also collected from each of the four cell lines mentioned above as well as from untransfected RPMI 8226 and Raji cells to identify total histone H1 proteins. In addition, whole cell lysates from the two non-transfected cell lines, mouse 10T1/2 and 2H1, were analysed for comparison of normal H1. For each cell type, cells were grown to 90% confluency/concentration. 10 mls of cells were then centrifuged and the resulting cell pellet was resuspended in PBS and centrifuged again. The final cell pellet was resuspended in SDS-PAGE sample buffer, boiled for five minutes, sonicated at maximum sonication for 20seconds and run on a 15% SDS polyacrylamide gel.

SDS Polyacrylamide gel electrophoresis

For SDS PAGE, the column samples were combined with an equal volume of 2x SDS sample buffer (125 mM Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.01% bromophenol blue). The samples were boiled for 1-2 minutes and immediately loaded onto the gel. Duplicate gels were run for each

sample, one for Coomassie Blue staining and one for immunodetection. A discontinuous two-part gel system was used. Each gel was 8 cm high, with approximately 6 cm of separating gel. For 2 ml stacking gels, 1.4 ml acrylamide + H_2O was added to 0.5 ml of 0.5 M Tris-Cl, pH 6.8, 20 µl of 10% w/v SDS, 20 µl 10% w/v ammonium persulfate, and 2 µl TEMED. For 7 ml separating gels, the appropriate amount of acrylamide stock was added to 1.75 ml 1.5 M Tris-Cl pH 8.8, 70 µl 10% w/v SDS, 40 µl 10% w/v ammonium persulfate, and 3.5 µl TEMED and made to a final volume of 7 mls with H_2O. The gels were run in a minislab apparatus (Hoefer miniVE Vertical Electrophoreis System; Amersham Pharmacia Biotech) at a continuous 170 V until 30 minutes after the dye front had run off the gel. The electrophoresis tray buffer consisted of 200 mM Tris, 1.52 M glycine and 0.4% SDS.

For primary identification, proteins were fixed and stained overnight at room temperature with 0.04% Coomassie Brilliant Blue G250 in 45% methanol and 9% acetic acid. The were first destained in 25% methanol, 12.5% acetic acid for approximately two hours with two changes of destain. They were further destained in a weak solution of 5% methanol and 7.5% acetic acid and were stored in this solution. The gels were then imaged wet on an Ultra-LUM Dual Light Transilluminator to identify fractions containing the histone H1 proteins.

The identity of H1.1-GFP was confirmed by immunoblotting. The proteins were transferred to Nitrocellulose membrane using a Hoefer miniVE blot module. All

protein detection was performed following the Amersham Pharmacia Biotech western blotting detection system (RPN 2132) ECL+Plus protocol. Total H1 primary antibody was used at a 1:1000 dilution. Anti-rabbit HRP at 1:10,000 dilution was used as the secondary antibody.

Section 2.4 Confocal Microscopy

2.4.1. Fixed Cell Confocal Microscopy

Adherent cell lines were trypsonized, seeded at low density onto glass coverslips (sterilized in 95% EtOH) and grown at 37°C until ~90% confluent. The cells were then fixed in 1% paraformaldehyde for 5 minutes and washed with PBS. Cells were permeabilized in PBS-T(Triton-X 100), 0.5% for 5 minutes. They were then rinsed three times in PBS. The cells were then incubated on 1° antibody by placing the coverslips upside down on a 25 µl drop of antibody mix that was placed on parafilm. After approximately one hour, coverslips were collected and rinsed twice in PBS-T (0.1%) and twice in PBS. Incubation with 2° antibody (conjugated to Cy3) was the same as with the 1° and similarly washed. Coverslips were then mounted onto glass slides containing a 10 µl drop of mounting media (1mg/ml para-phenylene diamine in 90% glycerol, in PBS and 30 ug/ml of the DNA dye, DAPI (Sigma Prod. no. D9542). Excess mounting media can be removed by dabbing a piece of 3MM filter paper on top of coverslips. Images were then taken on a Zeiss Axioplan 2

microscope with a 12-bit cooled Sensican CCD camera. 2-Dimensional images are shown through the mid section of the cell.

2.4.2. Live Cell Confocal Microscopy

Adherent cells for live imaging by confocal microscopy were trypsonized, seeded and grown overnight on glass coverslips sterilized with 95% ethanol, as above. Suspension, 8226 and Raji, cells were plated at low density onto glass coverslips coated with 1mg/mL Poly-L-lysine and grown for 24-48 hours until ~90% confluent. Individual coverslips containing cells were mounted with cell culture media onto glass slides and sealed in vacuum grease to minimize evaporation. A Zeiss Axiovert 510 confocal laser scanning microscope (LSM) with a 25 mA argon laser was used to perform all live cell experiments under a 40x multiimmersion objective. DIC/Normarski images were used to follow viability of cells. GFP confocal images were obtained using the Argon laser exciting at 488 nm at ~1% laser power. Images were taken at a bin number of 1.0 with the pinhole at 1.0 and scaled to 0.1um/pixel.

2.4.3. Fluorescence Recovery After Photobleaching (FRAP)

Each of the ten H1-GFP constructs (eight H1.1 and two H1.2) stabley transfected into both SK-N-SH and A549 cells, were imaged as stated above on the Zeiss laser scanning microscope. FRAP experiments were performed by exposing defined regions of cells to 100% laser intensity for typically 10-20 iterations. Imaging was

typically performed at 1% laser intensity as defined above. The interval between image scans varied depending on the duration of recovery in an initial pilot experiment. The interval between scans was set so that between 75 and 90 image scans were required in total for the experiment. Recovery was considered complete when the intensity of the photobleached region stabilized (i.e., the curve flattened). For quantitative analysis, fluorescence intensity was measured at each time point for 1) the photobleached region, 2) the entire cell nucleus, and 3) extracellular background intensity. Background was subtracted from each data point. To determine the percent recovery and account for the small amount of fluorescence loss due to repeated scanning during recovery, the measured whole nucleus fluorescence was used to normalize each data point to the fluorescence intensity of the nucleus obtained in the first image acquisition following photobleaching. The mobile population (percent recovery) was determined by dividing the normalized photobleached region at maximal recovery by the corrected (initial photobleached region intensity multiplied by total nuclear fluorescence immediately following photobleaching divided by total nuclear fluorescence immediately prior to photobleaching) fluorescence intensity of the photobleached region prior to photobleaching. Due to the contribution of electronic noise in the detector system, some measured recoveries were observed that were greater than 100%.

Empty vector-GFP, E-GFP, stabley transfected cells were similarly bleached with 80-100% laser intensity and imaged every second until recovered. The empty vector cell line required two times the number of iterations to ensure adequate bleaching of

the defined area. All quantitative analysis was performed using Metamorph Universal Imaging and Zeiss LSM 510 ROI software.

2.4.3.1. Histone H1 phosphorylation and FRAP

For energy-dependence experiments, cells were grown on glass coverslips prepared as before and treated with either 20 mM Sodium Azide (for 18-24 hours, 37°C) or 50 μM Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) with 200 mM 2-deoxyglucose for 4 hours at 37°C. The cells were then mounted onto glass slides in a small volume of media-containing drugs and sealed with vacuum grease. They were then bleached and imaged on the Zeiss 510 LSM as stated above. The cell recovery was followed for up to 45 minutes to monitor Histone H1.1-GFP movement into the bleached ROIs. For phosphorylation-dependence experiments, cells were treated with either 75 mg/ml 5,6-dichlorobenzimidazole ribozide (DRB) for 4-6 hours at 37°C with 2 μM Staurosporine for 4-6 hours at 37°C and bleached and imaged as above.

Chapter 3: Results. Characterization of Histone H1.1 Binding and Exchange

Section 3.1. Chapter Introduction and Experimental Objectives

During interphase, the higher-order folding of chromatin is known to be relatively stable at the chromosomal level. 3-D time-lapse imaging (4-D) of live cells shows minimal repositioning of DNA at the resolution of modern microscopy[145]. The reason for this stable positioning of chromatin is not well understood; however, the importance of it is becoming increasingly clear[145,146]. The identification of sub-chromosomal domains has recently given insight into the role that chromatin structure plays in regulating transcription, specifically in repressing it[147]. The classical dogma surrounding transcriptional activation is generally associated with the availability of specific transcriptional machinery, ranging from transcription factors to components involved in RNA processing. Now, a more non classical view suggests that both the spatial and temporal positioning of specific DNA sequences within the nucleus aids in regulating their transcriptional activation[148,149]. However, identification of the factors involved in modulating chromatin, as well as how they function *in vivo*, is not clearly defined. There are numerous DNA binding proteins, as well as associated factors, that are implicated in the stable folding and positioning of DNA during interphase. Controlled binding of both heterochromatin- and/or euchromatin-associated proteins could be the major determinants in both stabilizing and regulating DNA structure. In addition to identifying the proteins

involved in maintaining chromatin integrity, understanding how they function *in vivo* could have a profound impact on understanding the role that chromatin architecture plays in gene regulation. Answers to these questions may also have substantial influence on both understanding and possibly reversing conditions where there are dramatic chromosomal and chromatin abnormalities, such as those seen in cancer.

When DNA is synthesized or when small regions unwind, the naked DNA is lacking histones and thus does not retain a nucleosomal, "beads-on-a-string", structure. As histones H3 and H4 are deposited, the DNA begins to bind to, as well as wrap around, the histones[2]. Further association with histones H2A and H2B enables the approximately 146 bp of DNA to wrap two turns around the histone octamer core and thus form the nucleosome[2]. At this point, the kinked chromatin fiber is approximately 10 nm in diameter. It is not until the subsequent binding of histone H1 to the nucleosome that this structure, now termed the chromatosome, is stabilized such that it is resistant to nuclease digestion. The binding of histone H1 also drives and stabilizes the folding of the 10 nm chromatin fiber into the higher-order 30 nm fiber[82,88,103]. Addition of histone H1 is also critical, *in vitro,* for maintaining nucleosomal spacing and translational mobility, and therefore further regulates chromatin structure[3,4]. For this reason, histone H1 is believed to be stably bound to the DNA and thus cause stable repression of chromatin unwinding. More specifically, histone H1 is thought to be more tightly bound in condensed chromatin regions, such as heterochromatin, for maintaining stable repression of transcription.

Until recently, studies on exchange of histone H1 have been restricted to *in vitro* fractionated chromatin. Experiments of these types result in two distinct chromatin classes; one that precipitates out of solution and one that remains soluble. These classes are frequently referred to as aggregation-prone and aggregation-resistant, respectively. Further investigation showed that the acetylation state of the chromatin regulated its solubility. Highly acetylated chromatin was soluble in physiological ionic strength buffers[150]. As euchromatin is extensively acetylated and heterochromatin is minimally acetylated *in vivo*, the two *in vitro* chromatin populations, soluble and precipitated, have been loosely compared to euchromatin and heterochromatin, respectively. *In vitro* results extensively showed that histone H1 has the ability to freely exchange between isolated chromatin fragments within minutes[136,139-141]. However, it was noted by some that it could not redistribute between the two different chromatin populations[140]. That is, it was freely mobile within both the soluble and precipitated chromatin fragments but could not redistribute between the two populations. Jin and Cole argued that this was because histone H1 required direct fiber-fiber interactions to exchange[141]. They propose that histone H1 is not able to move as a soluble intermediate but rather must "jump" from one nucleosomal segment to another. In addition, their hypothesis may be supported by findings that the rate of histone H1 exchange increases with increasing ionic strength and that increasing ionic strength results in an increased number of chromatin fiber-fiber interactions[142]. Alternatively, their results may have been seen due to altered *in vitro* properties of the histone or DNA or differences in function of associated binding factors involved in regulating chromatin structure.

Regardless, it is apparent that *in vitro* analysis of histone H1 exchange characteristics is limited by the experimental design and therefore may not be directly reflecting what is occurring within a living cell.

Both the ability and absolute requirement of histone H1 to stabilize higher-order chromatin structures throughout the cell cycle suggest that it would be stably bound. Transient binding of the protein seems inhibitory for it to function as both a regulator and stabilizer of chromatin structure and thus result in large-scale transcriptional repression. For this reason, and due to technical limitations for studying living systems, there has been little emphasis directed at understanding histone H1 mobility *in vivo*, until the cloning of green fluorescent protein (GFP)[151]. The GFP reporter molecule is a bright, stable, non-toxic fluorophore. It is useful for imaging live cells as it bleaches (loses fluorescence) irreversibly under conditions unlikely to be found within living cells and does not bleach significantly under low-intensity microscopy imaging conditions. This enables proteins to be 'tagged' fluorescent green in live cells expressing the GFP fusion protein. In 1999, Gunjan and colleagues conducted the first documented studies of histone H1-GFP fusion proteins[144]. They successfully expressed this protein in a number of stable cell lines and analyzed what affects, if any, GFP had on histone H1 function. Upon examination with nuclease digestion experiments, they found that the GFP-tagged protein appeared to stabilize the 146 bp nucleosomal structure in a manner similar to native histone H1[144]. Through micrococcal nuclease and nucleoprotein gel experiments, they further showed that the GFP did not interfere with histone binding to the DNA[144].

Investigating the dynamics of histone H1 exchange, as well as what regulates its binding, is essential to understanding the role that this protein plays in maintaining chromatin integrity. In our studies, we have used human histone H1-GFP fusion proteins stably transfected into SK-N-SH, A549, 8226 and Raji human cell lines to characterize the binding dynamics of human histone H1 in living cells. *We provide evidence that the histone H1-GFP fusion protein is a reliable method for measuring histone H1 exchange properties in live human cells. We also demonstrate that greater than 95% of histone H1 is not stably bound to DNA in living cells and rapidly exchanges throughout the entire chromatin population of the nucleus. We further illustrate that direct fiber-fiber contact is not a prerequisite for histone H1 exchange and that exchange occurs through a soluble intermediate and is dependent upon the phosphorylation status of the cell.**

* A version of this chapter has been published in Lever et al (2000) *Nature*, **408**:873-876

Section 3.2. Binding of Histone H1.1-GFP fusion protein on chromatin of live SK-N-SH, A549, 8226 and Raji transfected cells

3.2.1. Does the GFP tagged histone H1 bind to DNA in a manner similar to native histone H1?

We have shown that histone H1.1-GFP binds to DNA in a manner similar to native histone H1.1 during interphase in living cells. Importantly, we have also demonstrated that the fusion protein remains bound throughout chromatin condensation prior to and during mitosis as well as into the next cell cycle. To illustrate the histone H1.1 binding pattern in SK-N-SH, A549, 8226 and Raji cells at different stages of the cell cycle, H1.1-GFP was stably transfected into each of the cell lines and fluorescence microscopy was performed on individual living cells. Cells were seeded at low density on glass coverslips and grown at 37^0C until approximately 85% confluent. Cells were then mounted in media containing the DNA dye, Hoechst. Using the 488nm Argon laser of the Zeiss laser scanning microscope, as well as a UV laser for Hoechst recognition, direct imaging of the GFP-tagged protein was analyzed concurrent with DNA imaging. 2-dimensional pictures of individual cells were recorded throughout a cell cycle. In all cell lines stably expressing H1.1-GFP, the histone H1 was seen to localize to the nucleus with little to no fluorescence in the cytoplasm (Figure 1). The small amount of extrachromosomal fluorescence (for example, see the telophase cell) is due to an autofluorescent component of these cells that emits at a slightly shorter wavelength than GFP, as determined by visual inspection, and is therefore not derived from H1.1-GFP. The similar expression patterns for both the Hoechst (and DAPI) and GFP, as well as the lack of unbound H1.1-GFP, demonstrates that the GFP tag does not appear to affect the normal nucleosomal binding of histone H1 in living cells.

To further test for any effects the GFP may have on histone H1 nucleosomal binding,

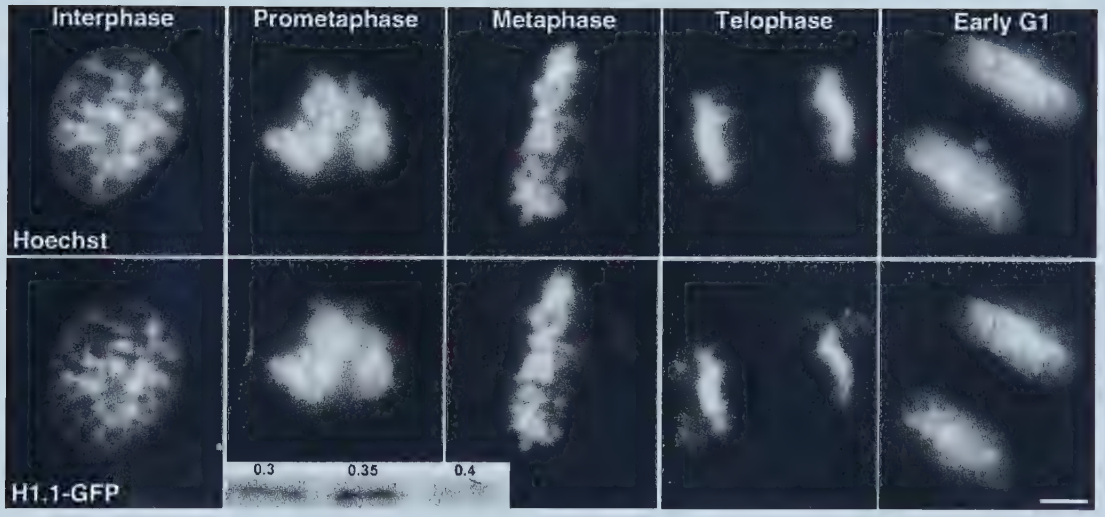

Figure 1. *Cell cycle distribution pattern of Histone H1.1-GFP bound to the DNA of live SK-N-SH transfectant cells.* Live cells stably expressing H1.1-GFP are stained with the DNA dye, Hoechst, and mounted onto glass microscopy slides. 2-dimensional FITC (for GFP) and UV (for DNA) images of cells are taken through the widest section of the nucleus at various stages of the cell cycle. Identical staining patterns of GFP and DNA, as well as the minimal unbound GFP present, indicates that the GFP does not interfere with normal histone H1 nucleosome binding. Scale bar, 5 mm.

we asked whether the GFP alone binds exclusively to DNA and thus aids in the recruitment of the H1.1-GFP complex to both the nucleus and chromatin. To answer this question, we transfected empty vector-GFP (EGFP) into the above cell lines. We show that the GFP signal was expressed randomly and uniformly in the cytoplasm with a stronger signal in the nucleus, possibly due to a slower rate of export out of the nucleus (Figure 2.) The signal present in the cytoplasm provides more evidence that it is histone H1 in the H1.1-GFP complex that is binding to chromatin in the transfected cell lines.

We further demonstrated the proper assembly of histone H1.1-GFP into chromosomes by measuring its salt-elution properties. We asked whether chromatin fragments from H1.1-GFP transfected cells would have altered affinity for hydroxylapatite columns in comparison to normal histone H1. Typically for these experiments, micrococcal nuclease-generated soluble chromatin fragments are bound to hydroxylapatite in the presence of 0.1 M phosphate. Buffers containing increasing NaCl concentrations are then run over the column. Under these conditions, the association of chromatin with the matrix is maintained by the DNA, while NaCl dissociates the histones from the chromatin. By this method, we are able to measure at what salt concentration specific histones are released from the chromatin. Multiple column fractions are collected and protein analysis is used to identify target proteins. Extensive studies of histone binding to hydroxylapatite columns have shown that typically total histone H1 proteins release from chromatin between 0.3 and 0.45 M NaCl, the histone H2A-H2B dimers elute at roughly 0.8 M NaCl and the

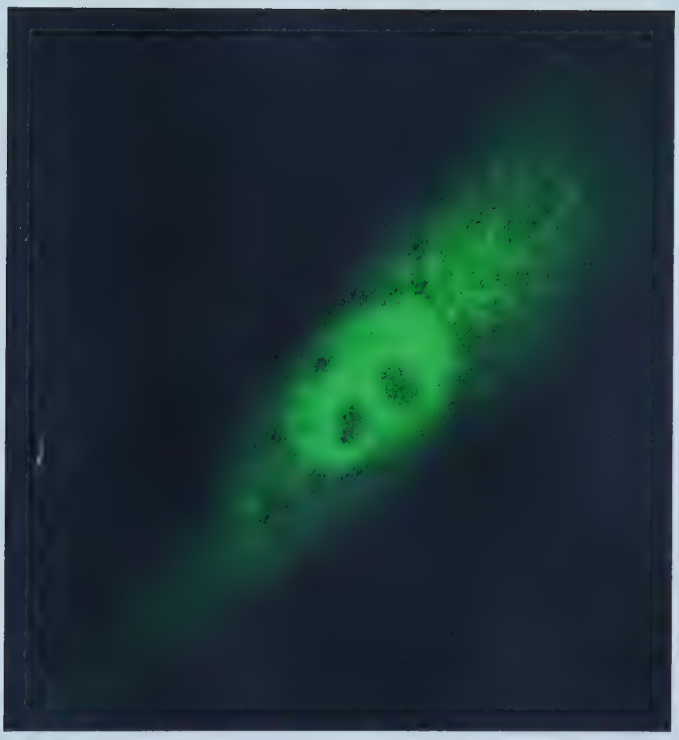

Figure 2. Localization and binding of empty vector-GFP (EGFP) in SK-N-SH cells. Live cell 2-dimensional imaging of EGFP stably transfected into SK-N-SH cells show that the GFP is randomly and uniformly distributed within the cytoplasm and the nucleus. The GFP signal present in the cytoplasm provides strong evidence that GFP itself does not act to localize the H1.1-GFP fusion protein to the nucleus or facilitate its binding to DNA. The enhanced signal in the nucleus is most likely due to a slower rate of export out of the nucleus.

histone H3-H4 tetramers elute at around 1.2 M NaCl[152]. In order to examine this *in vitro* binding property on GFP-tagged histone H1s, approximately 5 x 10^6 cells were grown and collected. Cells were then gently lysed to release intact nuclei. Following nuclei separation, they were lysed to allow isolation of chromatin. The DNA from each cell line was individually applied to hydroxylapatite columns and multiple fractions were collected. To identify which fractions contained the histone H1.1-GFP, proteins were run on an acrylamide gel and stained with Coomassie Blue. Western blot analysis was performed using a total histone H1 primary antibody to confirm the identity of the fusion protein. Fractions containing histone H1.1-GFP were pooled, as were the remaining fractions. Normal histone H1 runs near the 30 kD protein marker and H1.1-GFP is known to run just above 50 kD. In each case the histone H1.1-GFP was released between 0.3 and 0.4 M NaCl, similar to native H1 histones. As seen in the inset in Figure 1, the peak elution fraction of the approximately 50 kD H1.1-GFP protein occurs once the buffer salt concentration was increased to approximately 0.35 M. Identical results were seen with 8226 and Raji transfected lymphocytic cell lines. The presence of the eluted H1.1-GFP between 0.3 and 0.4 M NaCl, with a peak elution at 0.35M, demonstrates the ability of the fusion protein to bind with similar affinity as native histone H1. Therefore, we conclude that the H1.1-GFP fusion protein expressed in stably transfected cell lines is a reliable model for examining histone H1 binding and exchange in living cells. In addition, Misteli et al., have characterized the binding of several mouse histone H1-GFP variants[154]. Using micrococcal nuclease digests, salt elution experiments and

HPLC profiling, they have also shown that the GFP tag minimally interferes with binding of the histone H1s[154].

Section 3.3. Rate of Histone H1.1-GFP exchange in live SK-N-SH, A549, 8226 and Raji transfected cells

3.3.1. Is histone H1 stably bound to DNA?

To analyze the dynamic properties of histone H1 exchange in living cells, time-lapse recordings of cells stably expressing H1.1-GFP fusion proteins were used in fluorescence recovery after photobleaching (FRAP) experiments. Photobleaching experiments require rapid switching between a low-intensity imaging mode (for GFP identification) and a high-intensity photobleaching mode (designed to disable the GFP fluorescence). Because of the unique properties of the GFP fluorophore, pulses of a high intensity argon laser can be used to irreversibly photobleach the fluorescent tag in a specified area of the cell without losing GFP fluorescence in the surrounding area or causing damage to the histone H1 and/or the remainder of the cell. Over time, any recovery of fluorescence into the photobleached area is thus due to movement of fluorescent H1.1-GFP from the unbleached area of the nucleus into the bleached region. It is generally held that photobleaching does not affect the structure of GFP chimaeric proteins. In these experiments, it is also important to remember that it is not the photobleached histone H1.1 that was being monitored. Rather, it

was the exchange of the surrounding fluorescent H1.1-GFP into regions that had been photobleached.

In all live cell analysis reported in this thesis, the experiments were performed at least in triplicate. That is, 10-15 cells were analyzed on each of two or more coverslips plated on a minimum of three different days. For all experimental values reported, two-2 μm regions were photobleached and measured in each cell nucleus. These sizes were determined to minimize overall bleaching of the nucleus and allow averaging for individual cells. 2-dimensional images were scanned every 10 seconds following photobleaching to monitor exchange of histone H1 until the cells recovered. Equilibrium (or recovery) was reached between the bleached and unbleached regions within 3 to 5 minutes for SK-N-SH cells (with similar kinetics for both condensed and uncondensed chromatin regions). An SK-N-SH cell seen in Figure 3, illustrates that movement of the histone occurred laterally from the unbleached to the bleached region. To better visualize the movement of fluorescence, we bleached one-half of the cell nucleus. The intensity profiles in Figure 3, demonstrate that the intensity of the unbleached region decreased as the bleached region intensity increased, showing that recovery of histone H1 occurred from the movement of H1.1-GFP molecules from the unbleached region into the area that has been bleached. To confirm lateral movement of histone H1 from the unbleached to bleached region, we ensured that the specified area was bleached through entirely so that no exchange was occurring from top to bottom allowing for more accurate data analysis. To ensure that we were measuring movement of

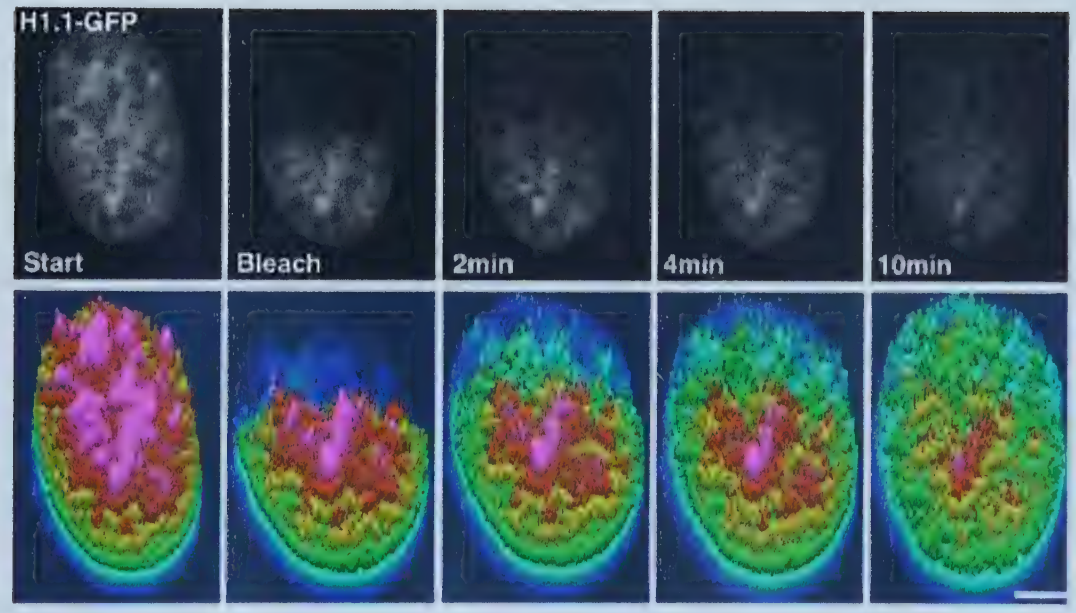

Figure 3. *Exchange of Histone H1 within nuclear chromatin of live cells.* The nucleus of an SK-N-SH cell stably expressing H1.1-GFP photobleached over a region covering roughly one-half of the nucleus. Movement of fluorescent H1.1-GFP was monitored until evenly distributed across the entire cell. Images were taken immediatelybefore and after photobleaching and at indicated intervals during recovery. The lower images represent a 3-D intensity profile of recovery of the cell in the upper panels. Pink indicates a higher intensity while blue represents minimal fluorescence. Scale bar, 5 µm.

existing H1.1-GFP molecules and to control for any refolding of GFP, *de novo* synthesis of GFP, transport from the cytoplasm or movement of H1.1-GFP from nearby cells, we performed subsequent experiments in which we bleached the entire nucleus of mono- and bi-nucleated cells. We consistently found that when an entire nucleus is bleached, there is no recovery of fluorescence.

3.3.2. Is the exchange rate of transfected H1.1-GFP faster or slower than other nuclear proteins?

In order to quantitate normal diffusion rates within the cells and to examine native GFP exchange, rates of GFP movement in empty vector-GFP (EGFP) stably transfected cell lines were monitored in similar photobleaching experiments as previously mentioned. The time-lapse photobleaching required twice the exposure time of the laser to significantly bleach through the entire cell, possibly due to the increase in GFP molecules. GFP reconstitution occurred over the entire cell extremely rapidly, approximately twenty times faster than the H1.1-GFP construct. Figure 4 shows a FRAP experiment of an SK-N-SH cell stably expressing EGFP. For illustrative purposes, approximately one-half of the nucleus and a small portion of the cytoplasm was photobleached (box). In the second image (immediately after photobleaching), the lack of distinct borders around the photobleached region demonstrates that the H1.1ΔC-GFP moves so rapidly that some protein enters and leaves the photobleached region during the duration of photobleaching (approximately 350 msec). These results suggest that this protein is unbound and

Figure 4. *Exchange of Green Fluorescent Protein within the nucleus and cytoplasm in vivo.* An SK-N-SH cell expressing empty vector-GFP photobleached over a region covering the upper half of the nucleus (box). Movement of GFP was monitored until evenly distributed across the nucleus. Images were taken immediately before and after photobleaching. The lack of a distinct photobleached region demonstrates that recovery was complete at the end of the photobleaching process, in approximately 400 ms. Scale bar, 5 μm.

moves rapidly by free diffusion.

To better interpret and understand our measured diffusion rate for histone H1, we also characterized nucleosomal histone, H2B, for comparison. Histone H2B-tagged GFP, when stably transfected into SK-N-SH cells, has a similar chromatin staining pattern as histone H1.1 (shown in Figure 1). This provides strong evidence that, like H1.1-GFP, the GFP tag does not interfere with binding of the H2B histone and therefore can be used as a reliable model for measuring H2B mobility. Interestingly, FRAP experiments performed under the conditions previously mentioned, demonstrated that this nucleosomal core histone is virtually immobile over a period of up to thirty minutes. In Figure 5, we see that when the bottom half of the nucleus was irreversibly photobleached, there was little recovery of fluorescence into the bleached region even after thirty minutes. The recovery curves seen in Figure 6 show the dramatic difference in rates of mobility for histone H1.1 compared to the nucleosomal histone, H2B, as well as unbound EGFP. On the Y-axis, the relative intensity ranges from 0 to 1.0. A value of 1.0 represents the theoretical percent recovery for the protein in question. That is, if 100% of the unbleached fluorescent proteins were able to move into the bleached region, the final intensity in the bleached area at equilibrium (when the intensity of all regions are equal) would be the value correlated with 100% mobility of the protein. By measuring the actual intensity of the bleached region at the time of equilibrium we can determine the percent of the protein that is available for exchange. For the unbound, empty vector-GFP we see that 100% of the protein is mobile and recovers instantaneously. For

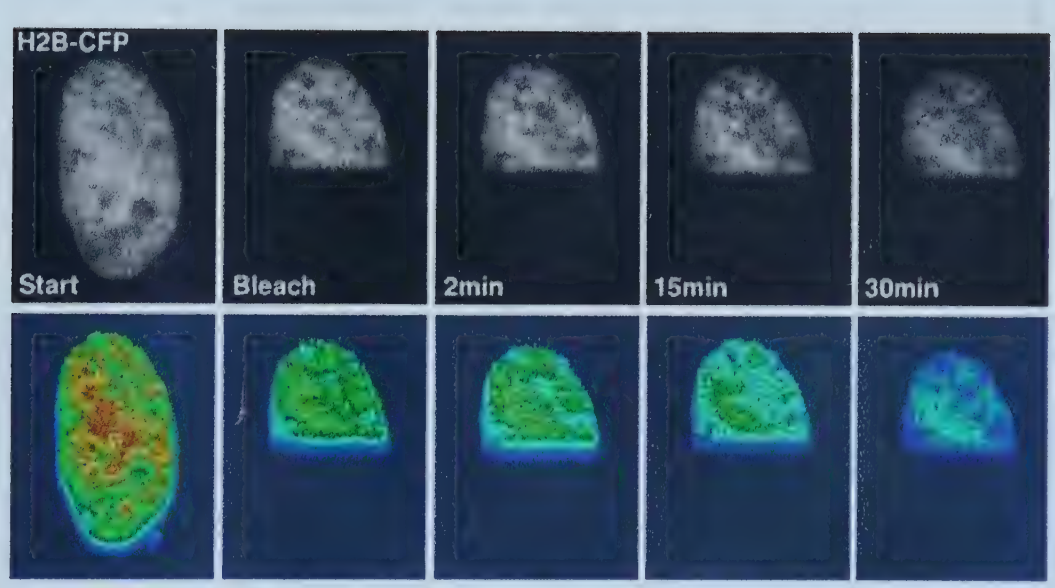

Figure 5. *Binding and mobility of Histone H2B in live SK-N-SH cells.* The nucleus of an SK-N-SH cell stably expressing histone H2B-CFP photobleached over a region covering roughly one-half of the nucleus. Recovery of fluorescence was monitored for 30 minutes. Images were taken immediately before and after photobleaching and at indicated intervals. The lower images represent a 3-D intensity profile of the nuclear fluorescence in the upper panels, with red being the highest intensity and blue the lowest.

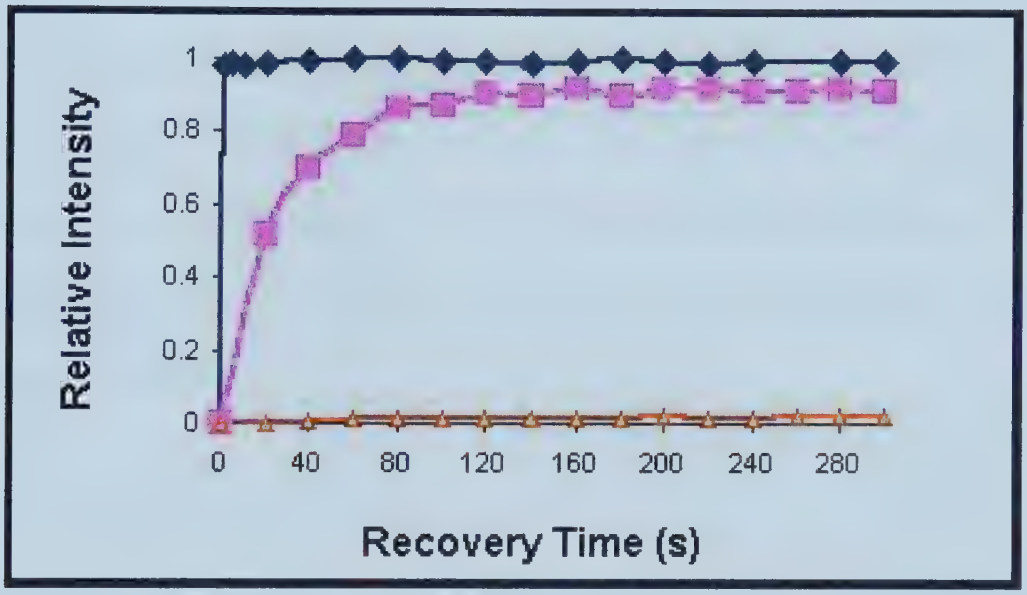

Figure 6. *Recovery profiles of spot bleaching experiments of histone H1.1 (purple squares) compared to histone H2B (yellow triangles) and the freely mobile, EGFP (blue diamonds).* To generate the curves, two 2-µm spots were photobleached simultaneously in each cell nucleus and fluorescence recovery was monitored quantitatively over time. The plot has been normalized so that the starting intensity (first image collected following photobleaching) represents 0 and the equilibrium intensity, reached at the end of recovery, represents 1.0. Thus, the initial values were greater than 1.0 and are not shown on the graph to better illustrate differences in recovery curves. The number of nuclei used to generate each plot was 34 for H1.1, 25 for H2B and 25 for EGFP.

histone H2B, the percent mobile population can not be calculated as the experiment did not run to equilibrium. Interestingly for histone H1.1, and in agreement with Misteli et al., there is a small, immobile fraction of the protein. This immobile population is measured to be less than 10% of the histone H1.1 and varies slightly as the energy status of the cell changes (see Section 3.4.3. and Table3-1 for variability). Here we provide evidence that histone H1.1 recovers rapidly in comparison to other DNA binding proteins, such as histone H2B, yet does not move as freely as the unbound protein, EGFP. In addition, it has been shown that histones H3 and H4 exchange slower than histone H1[137,138], as well as the chromatin binding proteins, SF2/ASF[153], HMG-17[154] and HMG-14[154], further demonstrating the rapid mobility of histone H1.

Section 3.4. Regulation of histone H1.1-GFP exchange in live SK-N-SH and A549 transfected cells.

3.4.1. Does exchange of H1.1-GFP require fiber-fiber interactions?

Previous studies by Jin and Cole supported a hypothesis that histone H1 requires contact between individual chromatin fibers in order for it to "jump" from one binding site to the next. This hypothesis is further supported by the fact that histone H1 is capable of crossing the nuclear envelope; however, in *in vitro* experiments in which the nuclei remain intact, there was no movement of the protein out of the

Table 3-1. *Time for 50% recovery of fluorescence and per cent mobile population of both untreated and drug-treated H1.1-GFP.*

Treatment	$t_{1/2}$ (s)	Mobile Population (%)
Control	18.7 ± 5.7 (n=68)	94.4 ± 7.7
Staurosporine	42.8 ± 18.8* (n=46)	91.7 ± 9.7
DRB	40.4 ± 20.8* (n=62)	93.0 ± 9.5
FCCP + 2DG	61.6 ± 24.0* (n=34)	81.6 ± 11.1*

The mean time for 50% recovery of fluorescence ($t_{½}$) and the per cent mobile population during the time course of the experiment was determined. For these data, more dense and less dense chromatin values were pooled. The statistical significance of the difference in recovery times relative to controls was determined by the Student's *t*-test.
*Probability that the means are equal is less than 0.0001.

nucleus. Experiments on histone H1 mobility to date do confirm the presence of a population of the protein that is capable of transferring from one chromatin binding site to another ('mobile fraction'). In general, i*n vitro* studies report a maximum of approximately 45% mobile fraction when studying radiolabeled histone H1[137,138]. However, it has remained unclear as to whether this DNA-binding protein dissociates from chromatin and travels through the nucleoplasm before resuming binding, or whether it requires fiber-fiber interactions as suggested by the *in vitro* studies mentioned previously. In order for us to examine the mode by which histone H1 exchanges from one chromatin fiber to the next in living cells, we made use of specialized cases that occur *in vivo*, in which isolated chromatin bodies are found. By examining lagging chromosomes during mitosis and isolated chromatin bodies during apoptosis, we could duelly determine which of the two possible modes of histone H1 movement are prominent *in vivo*.

Periodically, lagging fluorescent chromosomes are seen during mitosis in transfected cells (see the metaphase cell in Figure 7a). These chromosomes are physically isolated from the larger chromosomal mass and can thus be bleached to determine if histone H1 is capable of exchanging through an unbound population. FRAP experiments (Figure 7a), show that when the isolated chromosomes are bleached entirely, the fluorescence intensity fully recovers within 6-8 minutes (similar to kinetics of the larger chromosomal mass, Figure 7b). In addition, spontaneously arising apoptotic cells are occasionally seen in our experimentation. Phenotypically, early apoptotic cells possess small, condensed isolated chromatin bodies.

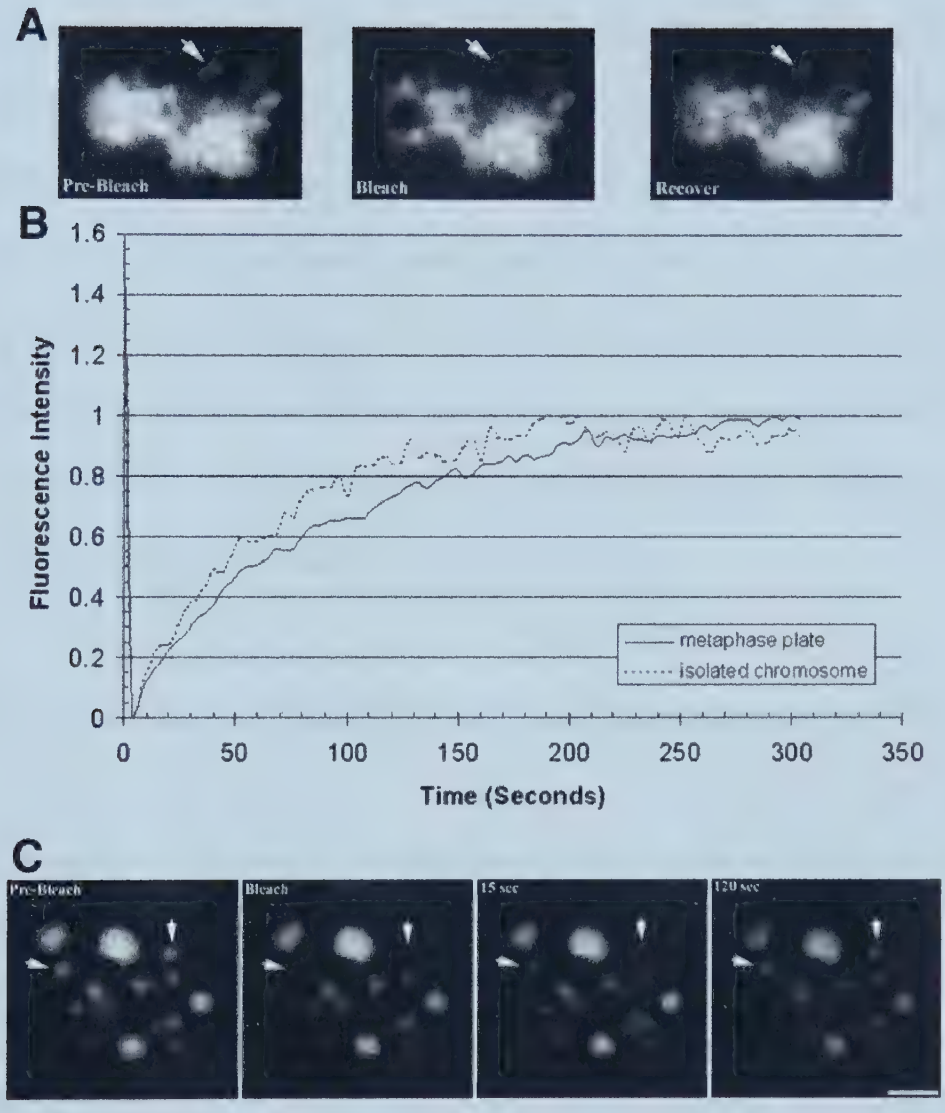

Figure 7. *Histone H1.1 exchange occurs between physically separated regions of chromatin.* a, A spot bleaching experiment on a metaphase cell containing a lagging chromosome is shown. Two spots were photobleached, one in the chromosome mass and the other containing the lagging chromosome (arrow). Images were taken immediately before and after photobleaching and at the indicated intervals. b, Relative intensity plot of the photobleaching experiment in a. c, A spot bleaching experiment on a spontaneously arising apoptotic cell. Two spots containing individual, isolated chromatin bodies (arrows) were photobleached and monitored for fluorescence recovery. Scale bar, 5 μm.

Again, photobleaching of this isolated chromatin provided an ideal template for determining if this histone was capable of exchanging through a soluble intermediate. FRAP experiments in which two apoptotic bodies were completely photobleached showed that both chromatin bodies fully recover fluorescence with kinetics similar to that of interphase chromatin movement of histone H1 (Figure 7c). Therefore, we clearly demonstrate that recovery of H1.1-GFP in both cases occurs despite isolation from unbleached chromatin during the course of the experiment. These results provide strong evidence that fiber-fiber interactions are not a strict requirement for the histone H1 exchange process *in vivo*, and further demonstrate that exchange occurs through a pathway that involves dissociation, diffusion through the nucleoplasm, and then reassociation with chromatin.

3.4.2. Is histone H1.1 exchange dependent upon the energy status of living cells?

In vitro studies of histone H1 mobility have demonstrated that exchange occurs in the absence of ATP. Moreover, H1 has been shown to fully redistribute in chromatin of isolated, intact nuclei as well as in highly fractionated chromatin. Thus, histone H1 does not require chemical energy to exchange *in vitro*. However, early into our experiments, we noticed a population of dieing cells that did not appear to recover as quickly as viable cells. This led us to believe that there may be an energy requirement for exchange of histone H1 *in vivo*. To test this, we employed the use of the mitochondrial poison, Sodium Azide (NaAz). NaAz acts to stop ATP production by inhibiting cytochrome oxidase c and ATP synthase, and hence kills the cell. Cells

were incubated with 20mM NaAz for up to 24hrs. FRAP experiments were performed as before with one-half of the nucleus bleached (Figure 8). Cell viability was followed using DIC images. The steady-state dynamics of nuclei containing the GFP chimeras were profoundly altered with NaAz. Time-lapse imaging of ATP-depleted cells revealed little to no exchange of the histone H1.1-GFP (Figure 8). Recovery of H1.1-GFP fluorescence was monitored over a longer time course to follow any slow movement of the histone. Treated cells showed a decreasing exchange rate with increasing exposure to NaAz. In completely necrotic cells, there was no recovery of fluorescence and hence no exchange, as seen in Figure 8.

To further assess if the energy status of the cell affected histone H1 exchange, we performed energy-depletion experiments on H1.1-GFP transfected cells and carried out FRAP experiments in conditions similar to those previously described. Our data suggests that the movement of H1 histones is not a strict energy-dependent process but rather is dependent upon the availability of phosphates. In order to moderately deplete cellular pools of ATP, we briefly (15-60 minutes) treated cells with the mitochondrial uncoupler, *p*-trifluoromethoxyphenylhydrazone (FCCP) + 2-deoxyglucose (2DG). Cells were grown to approximately 85% confluency on glass coverslips as previously described and drugs were added to the media in each well prior to mounting onto microscope slides. FRAP experiments show that the rapid mobility of histone H1 in the nucleus does not appear to be an energy-dependent process as the rate of recovery was not significantly affected after photobleaching in treated cells (data not shown). However, a reduced rate of exchange was seen when

Figure 8. *Exchange of H1.1-GFP within nuclear chromatin of Sodium Azide-treated cells.* **The nucleus of an SK-N-SH cell stably expressing H1.1-GFP is photobleached over a region covering approimately one-half of the nucleus. Movement of fluorescence is monitored for 30 minutes after treatment with the mitochondrial poison, Sodium Azide. Images were taken immediately before and after photobleaching and at 5 minute intervals. Scale bar, 5 μm.**

cells were treated with a longer incubation (4 hours) with these ATP-depleting agents (Figure 9). These results are consistent with the hypothesis that H1 movement is not dependent upon the availability of energy *per se* but on the phosphorylation status of the cell. That is, in brief FCCP + 2DG treatment, only moderate amounts of ATP are depleted from the cell and the activity of kinases and phosphatases are minimally affected. However, with prolonged treatment (prior to necrosis) the availability of phosphates decreases dramatically and the balance of kinase to phosphatase activity is largely shifted. This would lead to a decrease in the amount of phosphorylated proteins, including histone H1. Together, these results suggested to us that phosphorylation plays a role in histone H1 exchange.

3.4.3. Does phosphorylation regulate histone H1.1 binding and exchange?

Immunofluorescence examination with antibodies to phosphorylated histone H1 shows a reduction in staining of these cells after prolonged incubation with FCCP + 2DG but not after brief treatment. In addition, the prolonged treatment with these ATP-depleting agents significantly reduced the mobile fraction of histone H1 present in chromatin from 94.4 ± 7.7% to 81.6 ± 11.1% (see Table 3-1), which is consistent with our hypothesis that phosphorylation influences H1 binding. A failure to reach the predicted 100% recovery is assumed to reflect the presence of a population of photobleached molecules that can not be displaced from the photobleached region (that is, immobile population). It is therefore possible that post-translational phosphorylation of histone H1, or a protein that modulates its binding, may be

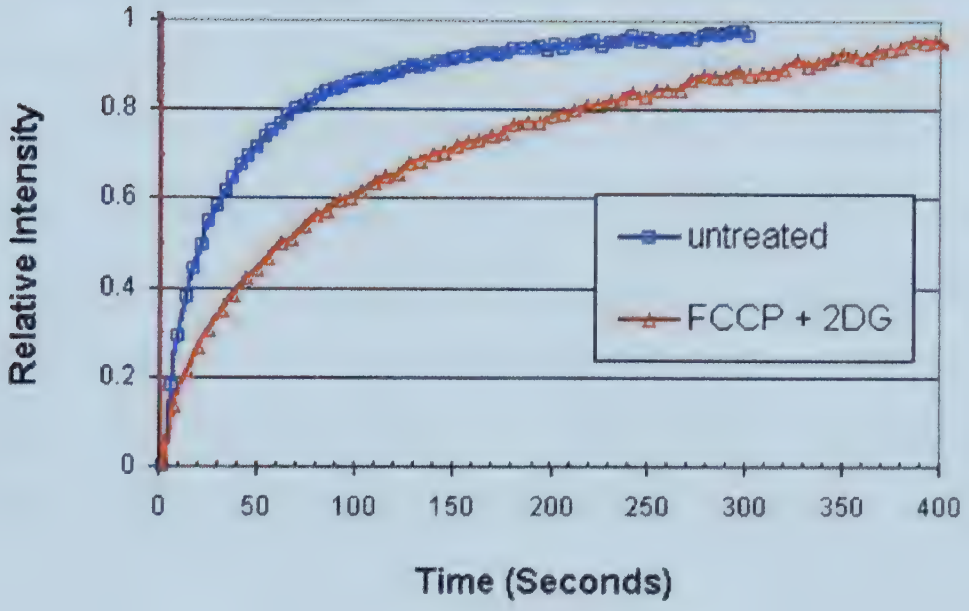

Figure 9. *Recovery profiles of spot bleaching experiments under untreated and prolonged drug-treated conditions.* Two 2-μm spots were photobleached simultaneously and fluorescence recovery was monitored quantitatively over time. The plot has been normalized so that the starting intensity (first image collected following photobleaching) represents 0 and the equilibrium intensity, reached at the end of recovery, represents 1.0. Thus, initial values were greater than 1.0 and are not shown on the graph to better illustrate differences in recovery curves. To generate the curves shown, two spots were simultaneously photobleached in each cell nucleus. The number of nuclei used to generate each plot was 34 for the untreated control and 17 for the FCCP + 2DG (4 hours) curve. For clarity, the FCCP + 2DG (brief treatment) curve was omitted.

involved in regulating the movement of histone H1.

To test this hypothesis, we used two general kinase inhibitors, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and Staurosporine. Both inhibit the phosphorylation of many proteins, including histone H1[96]. Cells were grown as previously described on microscope glass coverslips. Staurosporine or DRB was then added directly to the culture media. To avoid apoptosis, the cells were removed from the drugs within 4 hours of treatment and immediately monitored by FRAP imaging. As seen in Figure 10, when one-half of the nucleus of a drug-treated cell is photobleached, it requires roughly twice the amount of time to recovery as the untreated cell (Figure 3), suggesting that phosphorylation affects the exchange capability of histone H1. In Figure 10, we photobleached one-half of the nucleus to allow for easier visualization of protein movement. However, for all quantitative data reported, two, 2-μm spots were photobleached in all cells. As mentioned previously, photobleaching of the smaller spots (less than 10% of the nuclear volume) has been shown mathematically to be more accurate in measuring exchange of nuclear proteins. To further illustrate the effects of both Staurosporine and DRB, we plotted their recovery curves against the untreated control as well as FCCP + 2DG treated cells, seen in Figure 11. Cells treated with either kinase inhibitor showed an approximate doubling in the time required for 50% of the histone H1.1-GFP molecules to exchange ($t_{1/2}$; Table 3-1). The differences in the mean times required for 50% recovery of histone fluorescence were statistically significant when comparing drug-treated with untreated cells. These results strongly indicate that

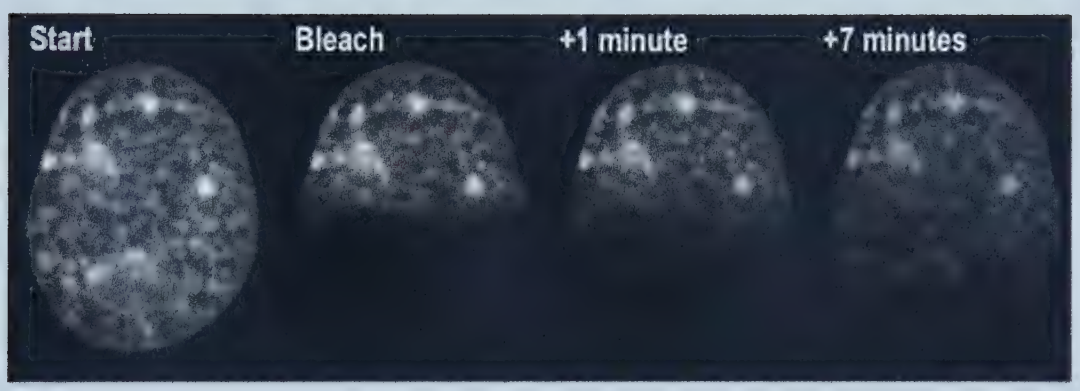

Figure 11. *Histone H1 exchange in vivo under treatment with kinase inhibitors.* A photobleaching experiment of an SK-N-SH cell stably expressing H1.1-GFP treated with the kinase inhibitor, Staurosporine. One-half of the nucleus was photobleached to follow recovery of fluorescence. Images were taken immediately before and after photobleaching and at one minute intervals until the end of the recovery period.

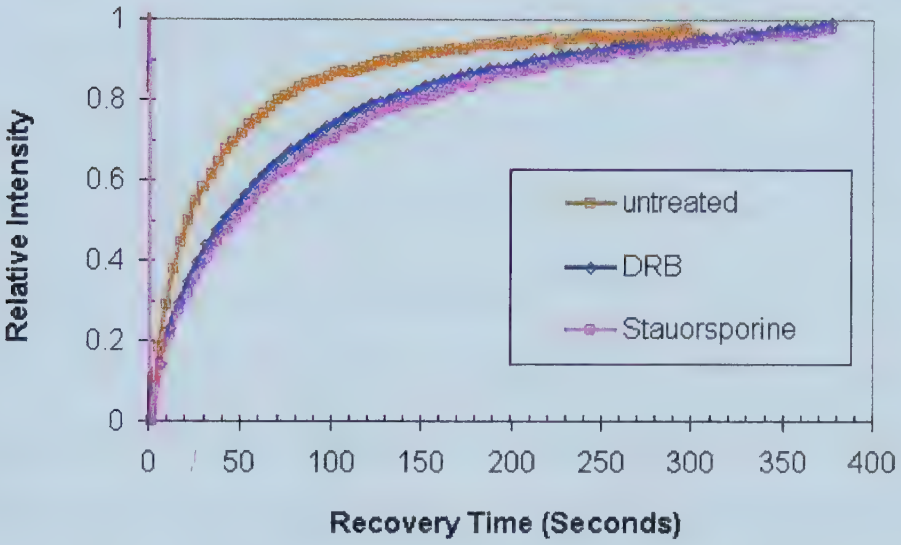

Figure 11. *Recovery profiles of spot bleaching experiments on III.1-GFP under untreated and drug-treated conditions.* Two, 2-µm spots were bleached simultaneously and fluorescence recovery was monitored quantitatively over time. The plot has been normalized so that the starting intensity (first image collected following photobleaching) represents 0 and the equilibrium intensity (reached at the end of recovery) represents 1.0. To generate the curves shown, two spots were bleached in each nucleus. The number of nuclei used to generate each plot was 34 for the control, 23 for the Staurosporine curve, and 31 for the DRB curve.

protein phosphorylation promotes the displacement of histone H1.1 from chromatin.

Section 3.5. Chapter Summary

Histone H1 is known to stabilize nucleosomes as well as the higher-order folding of chromatin. It is believed to be stably bound to DNA through two binding sites, one in the central globular domain and the other in the C-terminal tail; however, the exact binding locations remain unclear[77,78]. Histone H1 binding is generally thought to repress transcription, specifically to cause repression of a large number of genes through the stable folding of chromatin[3,4,105,106]. In this way, it can inhibit the access of transcription factors and chromatin remodelling complexes to DNA. Although these functions of histone H1 are well characterized *in vitro*, it is critical to determine its binding characteristics *in vivo* to better understand how it exerts these functions.

In SK-N-SH, A549, 8226 and Raji human cell lines, we demonstrated that GFP-tagged histone H1.1 is a reliable model for measuring the dynamics of histone H1 binding and exchange in living cells. We and others have extensively analysed both *in vitro* and *in vivo* binding characteristics of the H1.1-GFP fusion protein compared to native histone H1. H1.1-GFP stably transfected into cells has a chromatin binding pattern similar to native histone H1. Importantly, live cell imaging illustrated that this fusion protein remained bound to the DNA throughout the cell cycle. Thus, the fusion protein did not affect cell-cycle behaviour or cell proliferation. Furthermore,

when chromatin from cells stably expressing H1.1-GFP was applied to hydroxylapatite columns, the H1.1-GFP eluted from the column at the same salt concentration as native histone H1. This suggests that the GFP tag did not interfere with normal histone binding of the H1.1-GFP fusion protein. In addition, when empty vector-GFP was stably expressed in live cells, it did not localize to the nucleus. This further supports the idea that it is the histone portion of the H1.1-GFP that is binding the DNA and not the GFP. In total, we have provided strong evidence that the GFP tag minimally interferes with H1.1-GFP nucleosomal binding in living cells and can therefore be used to estimate the binding dynamics of histone H1 *in vivo*.

FRAP experiments performed on live cells stably expressing H1.1-GFP showed that histone H1 is dynamically associated with chromatin. When photobleached, the protein fluorescence recovered in less than 5 minutes. The mobility of histone H1 is extremely rapid compared to another nucleosomal histone, H2B, which demonstrated minimal exchange in up to 30 minutes. However, it did not appear to be freely diffusible as FRAP experiments performed on empty vector-GFP showed that this unbound protein recovers approximately twenty times faster than histone H1 (most likely representing free diffusion). Interestingly, quantitative analysis of fluorescence intensity during the FRAP experiments also found that approximately 5% of the histone population is immobile. To better understand how the mobile histone H1 population moves, we addressed the question as to whether or not fiber-fiber chromatin interactions are necessary for its to exchange. Contrary to what *in*

vitro studies suggest, we provide conclusive evidence that histone H1 moves through the nucleoplasm, from one binding site to another, as a soluble intermediate. Energy-depletion experiments using the mitochondrial uncoupler, FCCP, further support our hypothesis that histone H1 does not appear to have a strict energy requirement for exchange but more likely depends on the availability of phosphates. That is, brief treatment with ATP-depleting agents showed no affect on histone H1; however, prolonged treatment dramatically reduced its exchange rate. Interestingly, under prolonged treatment with FCCP + 2DG, the immobile population increased from approximately 5% to 20%, indicating that phosphorylation may stabilize histone H1 binding. To test this, we performed FRAP experiments on cells treated with one of two kinase inhibitors, both known to inhibit histone H1 phosphorylation. In both cases, the rate of exchange was approximately doubled. Thus, our results strongly argue that exchange of histone H1 *in vivo* is rapid, occurs through a soluble intermediate and is modulated by phosphorylation. However, at this point it remains unclear as to whether it is phosphorylation of histone H1 itself that regulates its exchange or phosphorylation of another protein involved in its binding and mobility. These questions will be addressed in Chapter 4.

Chapter 4: Results. Characterization of histone H1 phosphorylation and exchange using H1.1 N- and C-terminal Deletion Mutants and Histone H1.2

Section 4.1 Chapter Introduction and Experimental Objectives

The results reported thus far led us to revise our initial hypothesis as follows: *we propose that histone H1 is not stably bound, but rather transiently binds on and off of chromatin relatively quickly. More importantly, we show that phosphorylation of the histone dramatically alters its ability to exchange and suggest that it is phosphorylation of the C-terminal tail of histone H1 itself that is the major regulator of its binding and mobility.*

Direct phosphorylation of histone H1 has been shown to alter its association with chromatin[64]. It is believed that electrostatic repulsion between the phosphorylated histone H1 species and the DNA phosphate backbone weakens its binding. In accordance, interphase phosphorylation of histone H1, in contrast to mitotic hyperphosphorylation, is correlated with the transcriptionally active state of chromatin[133,134]. It is well accepted that the 'looser' phosphorylated histone H1 binding facilitates chromatin unfolding and allows transcriptional machinery to enter and bind. In addition, phenotype analysis in *Tetrahymena* indicates that phosphorylated histone H1 mimics H1 knockouts[100,155]. Thus, it is possible that phosphorylation of histone H1 may reduce its binding affinity for chromatin *in vivo* and thereby facilitate transcriptional activation.

Histone H1.1 has two p34cdc2/cyclin B kinase phosphorylation sites in its C-terminus as well as one site in the N-terminus that is phosphorylated only during mitosis[64,156]. Phosphorylation of the C-terminal tail is known to disrupt the interactions between this highly basic domain and DNA to alter chromatin organization[157]. It has therefore been suggested that phosphorylation of the C-terminus may have similar effects on histone H1 chromatin binding as with just the globular domain alone, as *in vitro* studies with reconstituted systems showed that the globular domain of histone H1 is sufficient to bind chromatin[85]. Notably, deletion of the highly positively charged C-terminus, which is the principle site of mitotic phosphorylation, may therefore have dramatic affects on the exchange rate of histone H1. However, deletion of the N-terminus, containing only one phosphorylation site, would not be expected to have as large an effect on histone H1 binding.

To test this, we first transfected SK-N-SH and A549 cells with an H1.1-GFP fusion protein in which the GFP was placed on the N-terminal tail of the histone rather than the C-terminus (as used in all previous experiments). Although we have reported that the H1.1-GFP retains similar nucleosomal binding characteristics of the native histone H1, we asked whether an intact C-terminus, not bound to GFP, would allow for more stable, 'normal' histone H1 binding *in vivo*. Any differences may therefore allow us to better understand how histone H1 binding is regulated. We additionally asked if treatment with kinase inhibitors would greater affect its mobility if the C-terminus was less obstructed by GFP. Furthermore, to better determine to what

extent the N- or C-termini play a role in histone H1 binding and exchange, we transfected individual H1.1-GFP fusion proteins in which either the N- or C-terminal tails were deleted. Each mutant was designed with the GFP replacing the deleted tail as well as with the GFP tagged to the opposite terminus. We stably transfected each construct (See Appendix I for protein sequences) into SK-N-SH and A549 cells and performed FRAP experiments as described previously. Quantitative analysis, using Metamorph Universal Imaging, allowed us to measure and compare the amounts that both the N- and C-termini of histone H1.1 contribute to stabilizing its binding.

Another histone H1 variant, H1.2, has four possible cdc2 kinase phosphorylation sites in the C-terminus and one in the N-terminal tail[156]. If histone H1 phosphorylation played a major role in its exchange, and not that of another phosphorylated protein(s), we proposed that histone H1.2 would be more sensitive to treatment with kinase inhibitors than histone H1.1 as it has two times the number of phosphorylated residues. Therefore, we stably transfected cells with H1.2-GFP and performed FRAP experiments as previously described. In addition, we characterized an H1.2-GFP fusion protein in which the N-terminus was deleted and measured the effects this had on histone binding and sensitivity to kinase inhibition. By doing this, we investigate the role that phosphorylation of the terminal tails play in regulating histone H1 binding and thus exchange. *We provide strong evidence that phosphorylation of histone H1, specifically of the C-terminus, plays a major role in regulating histone H1 binding and exchange for both histone H1 variants, H1.1 and H1.2. We provide further evidence that a specific phosphorylated amino acid at the*

end of the C-terminal H1.1 tail is the major regulator in stabilizing histone H1.1 binding within nuclear chromatin of live cells.

Section 4.2. Exchange rate and regulation of Histone H1.1 mutants

4.2.1. Does placement of the GFP tag on the N- or C-terminus affect the exchange rate of histone H1.1?

Until now, all data reported with H1.1-GFP has been with the GFP tagged to the C-terminal tail of the histone. As mentioned earlier, we chose to tag the GFP to the N-terminus to see what effects it may have on binding and determine if the C-terminus, not bound to GFP, would allow more stable binding of the protein. In this way, we can investigate the roles that placing GFP at both the N- and C-termini play in regulating binding of histone H1.

As seen in Figure 12, the chromatin staining pattern of the GFP-H1.1 is similar to that of H1.1-GFP. To measure the exchange rate and per cent mobile population of GFP-H1.1, we performed FRAP experiments on live cells stably expressing the fusion protein. Two, 2-μm spots were photobleached in the nucleus of each cell (Figure 12a). As before, the cells were imaged with Zeiss confocal laser-scanning microscopy until the fluorescence of the GFP-H1.1 was evenly distributed across the entire nucleus and the protein fluorescence had fully recovered. Surprisingly, we

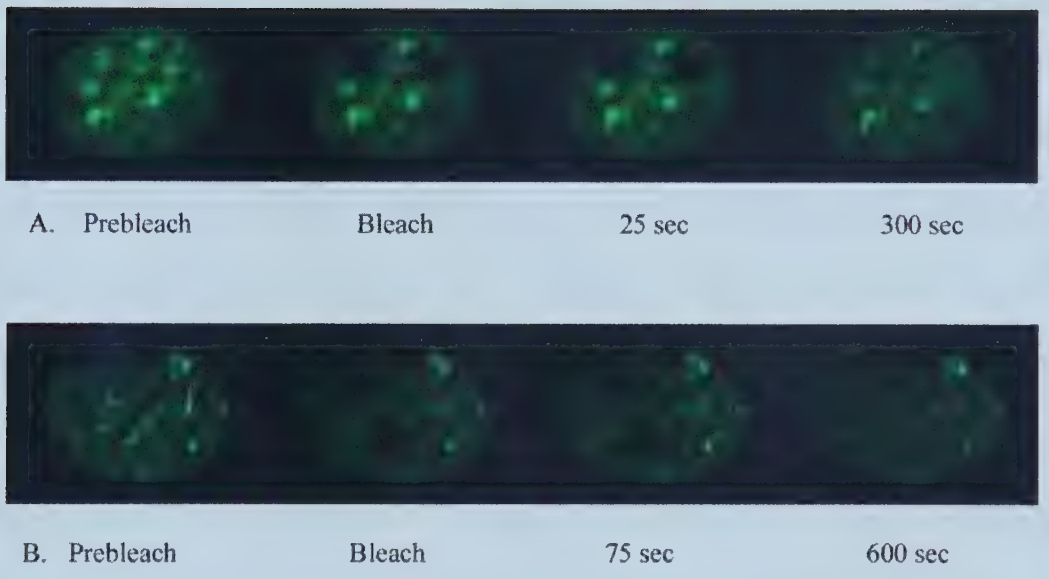

Figure 12. *Exchange of GFP-H1.1 in vivo.* A, an SK-N-SH cell stably transfected with GFP-H1.1 is photobleached in two, 2-µm spots. B, treatment of a GFP-H1.1-expressing SK-N-SH cell with 2 µM Staurosporine for 4 hours. In each case, movement of fluorescent GFP-H1.1 was monitored until evenly distributed across the entire nucleus. Live cell time-lapse images were taken immediately before and after photobleaching and at indicated intervals during recovery.

found that the movement of this fusion protein was reduced more than 2-fold when the GFP was tagged to the C-terminus, as demonstrated in Figure 13. Quantitative analysis of the fluorescence intensity over time showed that the $t_{1/2}$ for GFP-H1.1 is approximately 54 seconds, compared to 19 for H1.1-GFP (Figure 14). In addition, the per cent mobile population of the protein significantly decreased from 94.4% for H1.1-GFP to 89.6% for GFP-H1.1, suggesting that the unhindered C-terminus of GFP-H1.1 may be more stably bound (compare lines 1 and 3 of Table 4-1). Together, these results provide strong evidence that the C-terminal tail of histone H1 is largely regulating its binding and that the GFP tagged onto the C-terminus alters the dynamics of its exchange. Although it is apparent from our earlier experiments that the GFP tag does not significantly affect the ability of the histone to bind nucleosomes, it appears that it may influence the strength of binding. More importantly, what these experiments illustrate is that the C-terminus of histone H1 appears to be a major regulator in its binding to chromatin *in vivo*. That is, when the GFP is tagged to the N-terminus (and the C-terminus is therefore binding more similarly to how it natively binds) we see a dramatic stabilization of the protein resulting in a significant decrease in its rate of exchange.

To further characterize any differences between the GFP-H1.1 and H1.1-GFP, we performed FRAP experiments with Staurosporine-treated GFP-H1.1. Cells were grown, treated and imaged in conditions identical to those for H1.1-GFP. The chromatin staining pattern of GFP-H1.1 after Staurosporine treatment was similar to that of H1.1-GFP (Figure 12b). In addition, Table 4-1 shows that, like histone H1.1

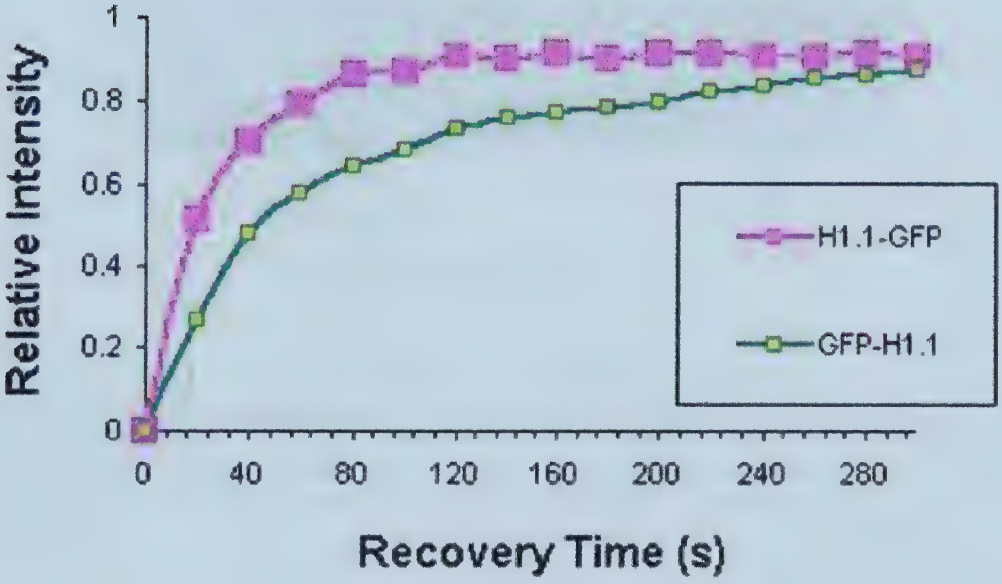

Figure 13. *Recovery profiles of spot bleaching experiments on GFP-H1.1 versus H1.1-GFP.* Two 2-μm spots were bleached simultaneously and fluorescence recovery was monitored quantitatively over time. The plot has been normalized so that the starting intensity (first image collected following photobleaching) represents 0 and the equilibrium intensity (reached at the end of recovery) represents 1.0. To generate the curves shown, two spots were bleached in each nucleus. The number of nuclei used to generate each plot was 68 for H1.1-GFP and 51 for GFP-H1.1.

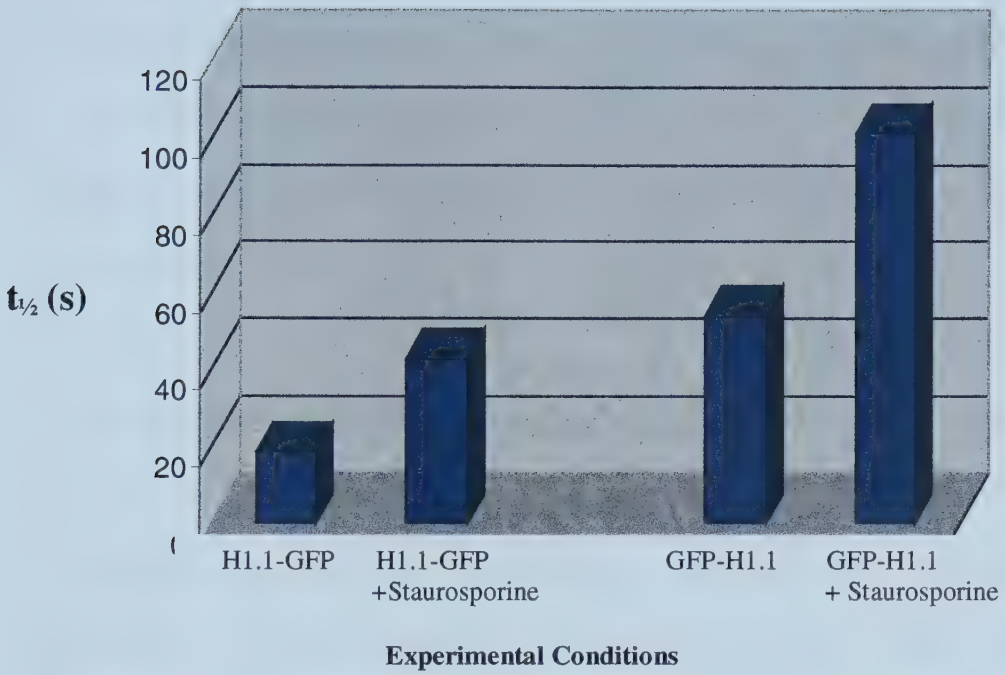

Figure 14. *Recovery times of GFP-H1.1 versus H1.1-GFP.* Quantitative analysis of FRAP experiments of cells stably expressing either construct. The time required for 50% of the fluorescent molecules to recover ($t_{½}$) are calculated from 68 H1.1-GFP untreated cells (46 Staurosporine-treated), and 51 GFP-H1.1 (36 Staurosporine-treated). Numerical values are reported in Table 2.

Table 4-1. *Time required for 50% recovery ($t_{½}$) and per cent mobile population of GFP-H1.1 versus H1.1-GFP.*

Treatment	$t_{1/2}$ (s)	Mobile Population (%)
H1.1-GFP	18.7 ± 5.7 (n=68)	94.4 ± 7.7
H1.1-GFP + Staurosporine	42.8 ± 18.8* (n=46)	91.7 ± 9.7*
GFP-H1.1	53.9 ± 9.4^{+} (n=51)	89.6 ± 4.7^{+}
GFP-H1.1 + Staurosporine	101.5 ± 20.8* (n=36)	97.5 ± 9.02*

*Probability that the means are equal is less than 0.0001 (comparing untreated to treated cells)
$^{+}$Probability that the means are equal is less than 0.0001 (comparing full-length H1.1 with the GFP tagged to N-terminus versus the C-terminus)

with the GFP tagged to the C-terminus, the amount of time required for 50% of the fluorescent GFP-H1.1 to recover after Staurosporine treatment, was approximately double that of the untreated controls. This suggests that although protein binding is more stabilized with GFP-H1.1, the relative influence of phosphorylation of the C-terminal tail (and consequences thereof) does not appear to be significantly disturbed by the GFP tag on either tail.

4.2.2. Does deletion of the N-terminal tail of histone H1.1 alter protein mobility?

To further investigate how phosphorylation affects histone H1 exchange and determine if it is phosphorylation of histone H1 itself that is a major regulator of its mobility, we transfected SK-N-SH and A549 cells with an histone H1 fusion protein lacking the N-terminus (referred to as ΔNH1.1). The N-terminus of H1.1 does not contain a DNA binding domain and is not believed to be phosphorylated during interphase. It is, however, believed to interact with the terminal tail of the core histones, H2A and H4, and we therefore expected that this N-terminal mutant would have different exchange dynamics than that of the full-length histone H1.

ΔNH1.1-GFP (GFP-tagged to the C-terminus) and GFP-ΔNH1.1 (GFP replacing the N-terminus) were similarly constructed. Each plasmid was stably transfected into SK-N-SH and A549 cells. Cells were grown as before on glass coverslips until approximately 85% confluent and mounted on glass microscope slides. Live cell imaging showed that each construct bound to chromatin in a manner similar to that

of the full-length histone H1. There was a small population of cells (less than 5%) that had some cytoplasmic fluorescence; however, the signal was extremely dim. This immediately suggested to us that these fusion proteins are not as stably bound to the DNA as the full length. Cells in which there was cytoplasmic staining were not included in the data collection.

FRAP experiments performed on each of the constructs showed that the N-terminal mutant moves more rapidly than the full-length histone H1. Figure 15 shows recovery curves of FRAP experiments on SK-N-SH cells stably transfected with ΔNH1.1-GFP. Two, 2-μm spots were bleached in each nucleus and imaged until fluorescence was fully redistributed across the cell. Quantitative analysis (summarized in Table 4-2) reveals that when the GFP was tagged to the C-terminus (ΔNH1.1-GFP) the immobile population was minimally affected; however, the $t_{1/2}$ of mobility significantly decreased to approximately 9 seconds (compared to the full-length control of 18.7 sec). Similarly, when the GFP replaced the N-terminus (thus the C-terminus is minimally affected), the $t_{1/2}$ was 12.1 seconds (compared to 53.9 for the full-length control; Table 4-2). These results demonstrate that the N-terminus does play a key role in stabilizing the histone binding. Although this tail is not believed to directly bind the DNA, it may be through its interactions with the core histone tails that it regulates histone H1 stability. However, and more importantly, these results indicate that the placement of the GFP tag on the C-terminus acts to weaken the histone binding and hence increase the exchange rate (from a $t_{½}$ of 12.0 to 9.1 seconds), indicating that the C-terminus is more highly affected by the GFP

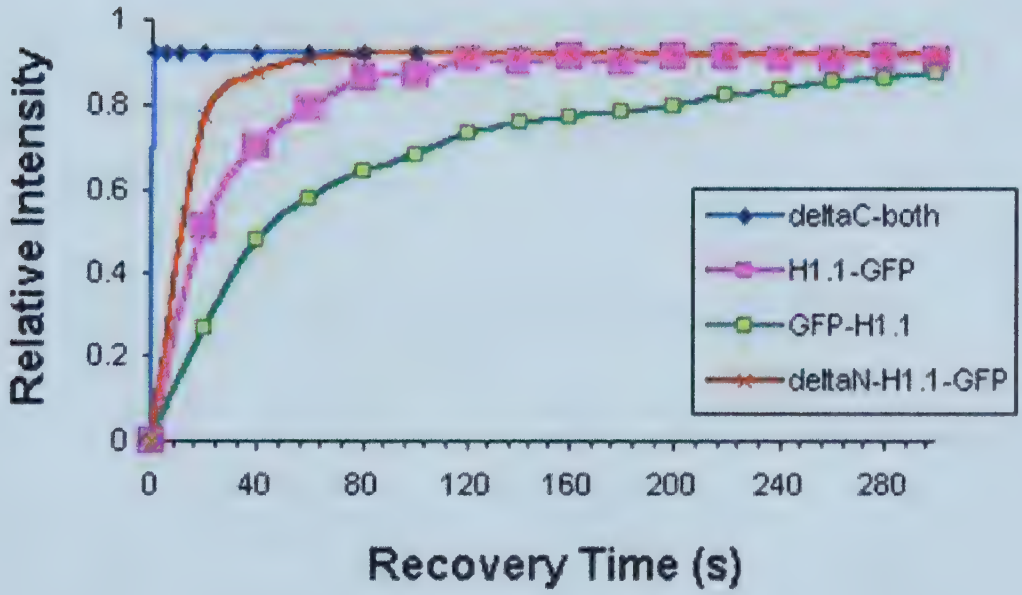

Figure 15. *Recovery profiles of spot bleaching experiments of histone ΔNH1.1-GFP compared to full-length H1.1-GFP, GFP-H1.1 and H1.1ΔC-GFP.* To generate the curves, two 2-μm spots were photobleached simultaneously in each cell nucleus and fluorescence recovery was monitored quantitatively over time. The plot has been normalized so that the starting intensity (first image collected following photobleaching) represents 0 and the equilibrium intensity, reached at the end of recovery, represents 1.0. Thus, the initial values were greater than 1.0 and are not shown in order to illustrate better differences in recovery curves. The number of nuclei used to generate each plot was 48 for ΔNH1.1-GFP, 68 for H1.1-GFP, 51 for GFP-H1.1 and 25 for H1.1ΔC-GFP.

Table 4-2. *Time required for 50% recovery ($t_{½}$) and per cent mobile population of ΔNH1.1-GFP in both untreated and drug-treated conditions.*

Treatment	$t_{1/2}$ (s)	Mobile Population (%)
ΔNH1.1-GFP	9.1 ± 3.0 (n=48)	95.8 ± 7.2
ΔNH1.1-GFP + Staurosporine	20.9 ± 7.7* (n=35)	101.9 ± 5.2
GFP-ΔNH1.1	12.1 ± 3.1^{+} (n=38)	96.4 ± 4.5
GFP-ΔNH1.1 + Staurosporine	26.9 ± 5.1* (n=30)	93.7 ± 5.8

Quantitative analysis of FRAP experiments of ΔNH1.1-GFP and GFP-ΔNH1.1 in both untreated and Staurosporine-treated conditions. Cells were treated with 3 μm Staurosporine for 4-6 hours.
*Probability that the means are equal is less than 0.005 (comparing untreated to treated similar cells)
$^{+}$Probability that the means are equal is less than 0.005 (comparing the GFP being tagged to the N- versus the C-terminus of the deletion mutant)

tag. In agreement with the experiments on the full-length H1.1 (Section 4.2.1.), these results strongly suggest that the C-terminal tail plays a more critical role in binding of histone H1 to chromatin than does the N-terminus. Staurosporine experiments further support our hypothesis that it is phosphorylation of the histone C-terminal tail that is the major regulator of its binding.

4.2.3. Does the phosphorylation status of the cell affect the N-terminal mutant of histone H1.1?

Although the N-terminus plays a substantial role in stabilizing histone H1 binding to chromatin, it is not expected to be affected by phosphorylation during interphase. Therefore, we predicted that treatment with kinase inhibitors would continue to affect the exchange rate of the GFP-tagged fusion proteins in a manner similar to that of their full-length counterparts. Thus, to further test whether the phosphorylation of the C-terminus is critical for histone H1 binding, we asked whether the N-terminal mutants are similarly affected by Staurosporine as are their full-length controls.

ΔNH1.1-GFP and GFP-ΔNH1.1 stably transfected cells were treated with Staurosporine as previously mentioned for the full-length H1 histones. FRAP experiments demonstrated that in both cases there was an approximate doubling of the time required for 50% of the fluorescent molecules to be fully redistributed across the nucleus. In addition, similar to Section 4.2.1., the N-terminal mutant with the GFP tagged to the C-terminus appeared to bind less tightly to chromatin as

demonstrated by the decreased $t_{1/2}$. Table 4-2 compares the $t_{1/2}$ and per cent mobile population for each construct in both treated and untreated conditions. The effects of Staurosporine treatment appeared similar, if not identical, to that of the full-length constructs. This is not surprising as the N-terminus was not expected to be affected by the phosphorylation status of the protein. These results do not however exclude the possibility that phosphorylation of another protein, aside from histone H1, is largely regulating its exchange.

*4.2.4. Is the mobility of histone H1.1 altered by deletion of the C-terminal tail?**

* A portion of this section is published in Lever et al (2000) *Nature*, **408**:873-876

To more thoroughly examine the role of histone H1 phosphorylation in its exchange capabilities, we deleted the C-terminus of the H1.1-GFP fusion protein which contains the two major sites of phosphorylation. The GFP was tagged to both the N- and C-terminus of H1.1 (GFP-H1.1ΔC and H1.1ΔC-GFP, respectively). We provide compelling data that strongly suggests that the C-terminal tail of histone H1 is the major regulator of its ability to bind chromatin. We stably expressed H1.1ΔC-GFP in SK-N-SH, A549, 8226 and Raji human cell lines. A small percentage of these cells had a phenotype similar to empty vector-GFP (seen in Figure 4). That is, green fluorescence was detected in the cytoplasm as well as the nucleus. However, in the H1.1ΔC-GFP transfected cells, a chromatin binding pattern was seen in the nucleus. This suggests that the C-terminal mutant is less tightly bound to the DNA than the full length fusion protein, but is still binding DNA. As it is well known that the

globular domain of histone H1 itself is sufficient to bind chromatin, this suggests that the C-terminus may be the site of regulation for increasing or decreasing the stability of protein binding. As seen in Figure 16, time-lapse microscopy of FRAP experiments performed on these cells, illustrate that the mutated histone moves extremely rapidly. Quantitative analysis reveals that the time required for 50% of the histone H1 to exchange was dramatically reduced, from 18.7 seconds to less than one second (Figure 17, Table 4-3). In addition, the mobile population significantly increased to approximately 100% (Table 4-3). Theses results strongly argue that the C-terminus of histone H1, with multiple sites of phosphorylation, is necessary for stable binding of histone H1 to chromatin.

4.2.5. Is the exchange rate of the C-terminal mutant affected by the phosphorylation status of the cell?

If phosphorylation of the C-terminal tail was the major regulator of histone H1 binding, we would expect that treatment with kinase inhibitors would have minimal influence on the exchange rate of H1.1ΔC-GFP. However, if phosphorylation of another protein, aside from the histone, was largely involved in modulating its binding and exchange, we would expect that treatment with kinase inhibitors on the C-terminal mutant of H1.1-GFP would have continued effect on the rate at which histone H1 redistributed. To help discriminate between these two possible modes of regulation, we treated the H1.1ΔC-GFP stably transfected cells with the general kinase inhibitor, Staurosporine. Cells were grown on glass coverslips until

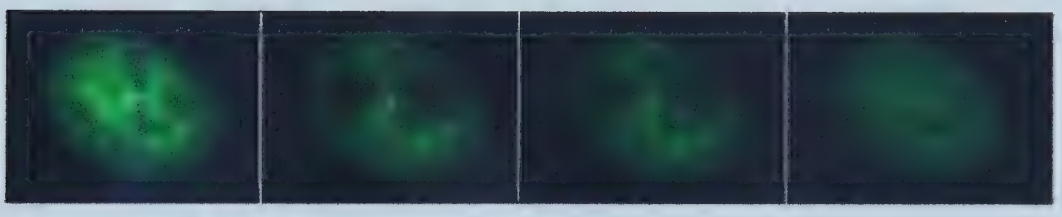

Figure 16. *Exchange of H1.1ΔC-GFP in living cells.* An SK-N-SH cell stably expressing H1.1ΔC-GFP (with GFP replacing the C-terminus) is photobleached in two, 2-μm spots. FRAP images are taken immediately before and after photobleaching (first two panels) and at 400 msec intervals. Movement of fluorescent H1.1ΔC-GFP was monitored until evenly distributed across the entire nucleus. Similar results were obtained with GFP-H1.1ΔC.

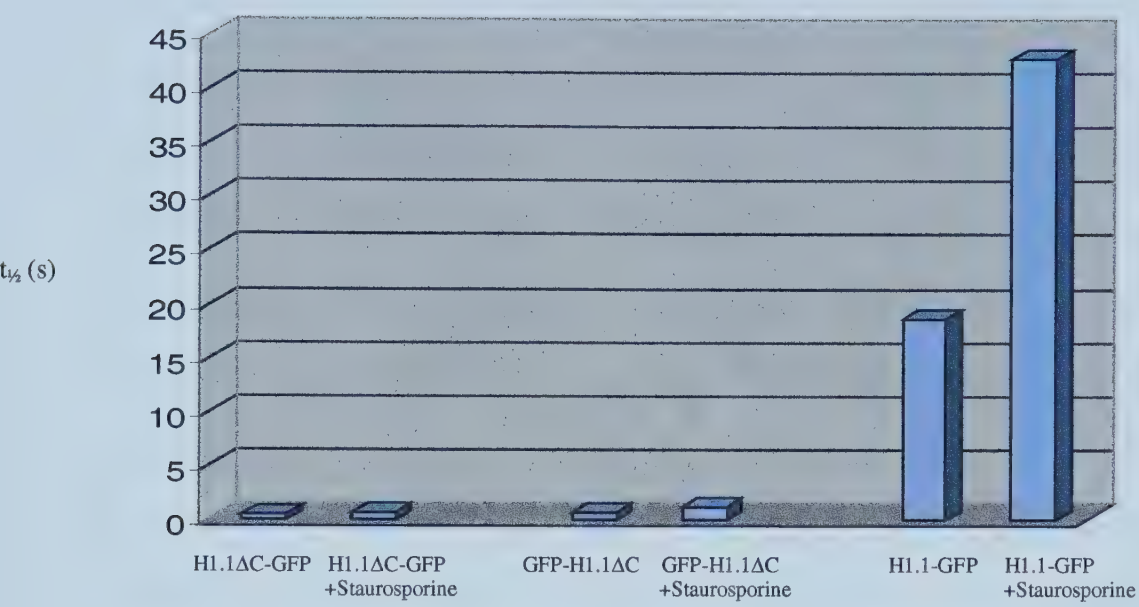

Figure 17. *Recovery times of C-terminal H1.1 deletion mutants.* Quantitative analysis of FRAP experiments of stably transfected SK-N-SH cells. The times required for 50% of the fluorescent molecules to recover ($t_{½}$) are calculated from 25 H1.1ΔC-GFP untreated cells (36 Staurosporine-treated), 68 GFP-H1.1ΔC untreated cells (44 Staurosporine-treated) and 68 H1.1-GFP untreated cells (46 Staurosporine-treated). Numerical values are reported in Table 2.

Table 4-3. *Time required for 50% recovery ($t_{½}$) and per cent mobile population of H1.1ΔC.*

Treatment	$t_{1/2}$ (s)	Mobile Population (%)
H1.1ΔC-GFP	0.49 ± 0.1 (n=25)	101.8 ± 4.3
H1.1ΔC-GFP + Staurosporine	0.58 ± 0.19 (n=36)	102.4 ± 5.8
GFP-H1.1ΔC	0.71 ± 0.2 (n=68)	103.2 ± 4.8
GFP-H1.1ΔC + Staurosporine	1.09 ± 0.34* (n=44)	106.7 ± 6.0

Quantitative analysis of FRAP experiments of H1.1ΔC-GFP and GFP-H1.1ΔC stably transfected cells. Values for both untreated and Staurosporine-treated cells are shown. Cells were treated with 3 μm Staurosporine for 4-6 hours.
*Probability that the means are equal is less than 0.005 (in comparison to untreated, similar cells)

approximately 85% confluent. Staurosporine was added for 4-6 hours to the culture media prior to live cell imaging. Again, cells were removed from the drug within 6 hours to avoid apoptosis. FRAP imaging of live cells showed similar exchange capabilities as with the untreated controls seen in Figure 17. Table 4-3 shows that, like the untreated H1.1ΔC-GFP transfected cells, the drug-treated cells completely recovered in less than one second and demonstrated 100% mobility of the protein. However, due to limitations in our Metamorph universal imaging system and resolution of our microscope, it is difficult to accurately measure exchange rates less than one second. Therefore, there may be moderate affects of Staurosporine on the C-terminal mutants of histone H1.1 that are unable to be detected. Regardless, we saw no significant increase in the exchange rate of H1.1ΔC-GFP stably transfected cells upon treatment with Staurosporine. This suggests that it is phosphorylation of histone H1 itself, and not that of another protein, that is regulating its binding.

Section 4.3. Exchange and regulation of the Histone H1 variant, H1.2, in live H1.2-GFP transfected SK-N-SH and A549 cells

4.3.1. Do histones H1.1 and H1.2 have different mobilities?

From the data presented thus far, it is apparent that the C-terminal tail of H1.1 is a critical component for stable binding of this protein to chromatin. It is also strongly suggested that phosphorylation, specifically of the C-terminus, regulates histone H1.1 binding and exchange. However, indirect immunofluorescence microscopy

suggests that some histone H1 variants may not have gene-specific association; therefore[91], their C-terminal domains may have a different function from other histone subtypes with gene-specific functions. In addition, localization studies of different H1 variants suggest that the subtypes of human histone H1 could have specific functions in the nucleus. We therefore chose to compare the binding dynamics of another histone H1 variant, H1.2, with H1.1. As stated previously, histone H1.2 has four possible phosphorylation sites in the C-terminal tail (Threonine 126, 146 and 154; Serine 173) and one in the N-terminus (Threonine 31). Histone H1.2 has been shown to have 93.6% sequence homology with H1.1, with the variation seen in large portions of the C-terminal tail, perhaps allowing for altered function. Histone H1.2 is less highly expressed than H1.1 and is also believed to interact with the terminal tails of the core histones. We asked whether histone H1.2, with four phosphorylation sites, would be less tightly bound to chromatin (due to electrostatic repulsion between the phosphates) and perhaps more sensitive to treatment with kinase inhibitors.

To test this, we stably transfected H1.2-GFP (GFP tagged to the C-terminus), into SK-N-SH and A549 cells. Live cell imaging showed that H1.2-GFP has a chromatin staining pattern similar to H1.1-GFP (Figure 18). We performed FRAP experiments on individual cells and measured the exchange rate and per cent mobile population of H1.2-GFP (Figure 18). Two, 2-μm spots were photobleached in each of a number of nuclei. Upon quantitation, we found that the exchange rate of H1.2-GFP was slightly faster than that of H1.1-GFP, although not significantly different to 0.005.

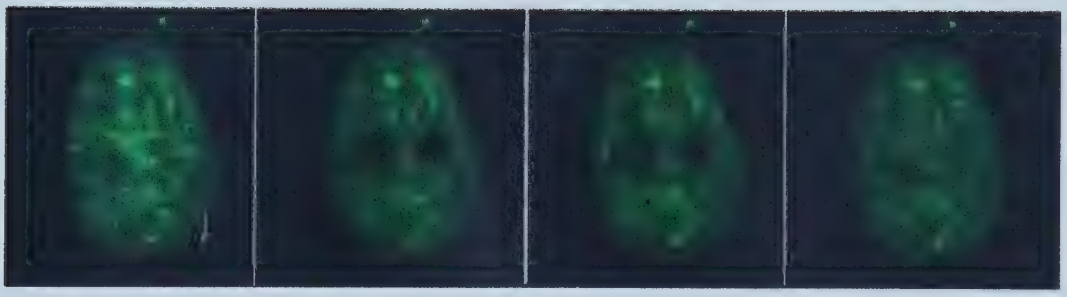

Figure 18. *Exchange of Histone H1.2-GFP within nuclear chromatin of live cells.* An SK-N-SH cell stably expressing H1.2-GFP photobleached in two, 2-μm spot within the nucleus. Movement of fluorescent H1.2-GFP was monitored until evenly destibuted across the nucleus. Images were taken immediately before and after photobleaching and at 15 second intervals during recovery.

The $t_{1/2}$s for H1.2-GFP and H1.1-GFP were 16.2 and 18.7, respectively (Table 4-4). The time required for 50% of the fluorescent molecules to redistribute suggests that histone H1.2 is not less tightly bound to chromatin than histone H1.1.

4.3.2. Are there differential effects of Staurosporine treatment on histone H1.2 versus H1.1?

The increased number of phosphorylation sites on the C-terminal tail of histone H1.2 may suggest that upon treatment with kinase inhibitors it would be more sensitive to exchange that treatment of histone H1.1. To test this, we treated H1.2-GFP with the general kinase inhibitor, Staurosporine and performed FRAP experiments. We saw a dramatic decrease in the rate of exchange of this histone after drug treatment. Using Metamorph Imaging, data analysis revealed that there was a 2.66 times decrease in the mobility of this protein, as compared to 2.29 for H1.1-GFP (Table 4-4). Although this demonstrated that, like histone H1.1, binding and exchange of histone H1.2 is significantly altered by phosphorylation, there is not significant difference in the influence of phosphorylation. That is, histone H1.2 (with four phosphorylation sites on its C-terminal tail) is no more affected by treatment with kinase inhibitors than is histone H1.1. This suggests that it may be only one or two specific phosphorylated residues that regulate histone H1 exchange and not an overall charge effect.

Table 4-4. *Time required for 50% recovery ($t_{1/2}$) and per cent recovery of histone H1.2 versus histone H1.1*

Treatment	$t_{1/2}$ (s)	Mobile Population (%)
H1.2-GFP	16.2 ± 3.6 (n=63)	101.9 ± 4.1
H1.2-GFP + Staurosporine	43.1 ± 8.9* (n=42)	96.6 ± 5.8*
H1.1-GFP	18.7 ± 5.7 (n=68)	94.4 ± 7.7
H1.1-GFP + Staurosporine	42.8 ± 18.8* (n=46)	91.7 ± 9.7

Values reported are averages from FRAP experiments of H1.2-GFP and H1.1-GFP stably transfected SK-N-SH and A549 cells. Both untreated and Staurosporine-treated cells have been used and number of nuclei (n) examined in each case is reported.
*Probability that the means are equal is less than 0.005

4.3.3. Does deletion of the N-terminal tail of histone H1.2 alter its exchange capabilities?

Similarly to studies on histone H1.1, we wanted to examine the role of the N-terminal tail of H1.2 in its binding. Like the terminal tails of H1.1, there is little known about their function. The C-terminus is believed to interact with DNA and allow for stable binding of the protein; however, less is known about the function of the N-terminal tail. Based on its structure, with one phosphorylation site, we suspect deletion of this tail would cause the protein to be more sensitive to treatment with kinase inhibitors than would the N-terminal deletion mutant of H1.1 with no phosphorylation sites in its N-terminus. It is important to note here that although we attempted to make transfectants of a C-terminal deletion mutant of H1.2, we were unable to successfully do so.

To examine the role of the H1.2 N-terminus in its binding and exchange, we stably transfected cells with ΔNH1.2-GFP and treated them with Staurosporine as previously mentioned for the full-length H1.2. FRAP experiments demonstrated that there was an approximate tripling of the time required for 50% of the fluorescent molecules to be fully redistributed across the nucleus. Table 4-5 compares the $t_{1/2}$ and per cent mobile population for ΔNH1.2-GFP in both treated and untreated conditions compared to that of the full-length H1.2-GFP as well as the N-terminal deletion mutants for histone H1.1 with the GFP similarly tagged to the C-terminus. As compared to ΔNH1.1-GFP with a 2.3 times increase in $t_{½}$, the increase of 3.1

Table 4-5. *Time required for 50% recovery and per cent mobile population of ΔNH1.2-GFP in living cells.*

Treatment	$t_{½}$ (s)	Mobile Population (%)
ΔNH1.2-GFP	6.6 ± 1.8 (n=46)	102.5 ± 3.7
ΔNH1.2-GFP + Staurosporine	20.4 ± 4.4* (n=38)	101.5 ± 5.3
H1.2-GFP	16.2 ± 3.6^{+} (n=63)	101.9 ± 4.1
H1.2-GFP + Staurosporine	43.1 ± 8.9* (n=42)	96.6 ± 5.8*
ΔNH1.1-GFP	9.1 ± 3.0 (n=48)	95.8 ± 7.2
ΔNH1.1-GFP + Staurosporine	20.9 ± 7.7* (n=35)	101.9 ± 5.2*

Quantitative analysis of FRAP experiments of the N-terminal deletion mutant of histone H1.2-GFP (ΔNH1.2-GFP) stably transfected SK-N-SH and A549 cells compared to full-length H1.2-GFP and the N-terminal deletion mutant of histone H1.1-GFP (ΔNH1.1-GFP). Data analysis is from untreated and Staurosporine-treated conditions. The number of nuclei (n) used in each case is reported.
*Probability that the means are equal is less than 0.0001 (when comparing untreated to treated cells transfected with similar fusion proteins)
$^{+}$ Probability that the means are equal is less than 0.005 (comparing full-length H1.2-GFP to the N-terminal deletion mutant of H1.2-GFP)

fold for ΔNH1.2-GFP suggests that this protein is more sensitive to Staurosporine treatment and thus the phosphorylated residue in the H1.2 N-terminus may therefore contribute to histone H1.2 binding and stability.

Section 4.4. Site-Directed Mutagenesis of specific histone H1.1 phosphorylated residues.

4.4.1. Does substitution of either Threonine 152 or Serine 183 to Alanine significantly affect the exchange rate of histone H1.1?

Once we had determined that the C-terminal tail of histone H1.1 was the major regulator of its binding and stability, we wanted to further investigate how it functions in this way. To examine the individual roles of each of the two phosphorylated amino acids in the C-terminal tail, we transfected cells with GFP constructs in which either of these residues was substituted to alanine. In this way, the mutated residues mimicked the non-phosphorylated state. In order to minimally affect binding of the C-terminus, the GFP was tagged to the N-terminal tail of each fusion protein. The first GFP construct in which only Threonine 152 is substituted to alanine, is referred to as GFP-T152A; the other with only Serine 183 substituted to alanine is referred to as GFP-S183A.

Quantitative analysis of stably transfected cells was used to determine the $t_{½}$ and per cent mobile population for each fusion protein. For GFP-T152A, there was a

substantial increase in the rate of exchange of the protein with approximately 100% of the protein being mobile (Figure 19, Table 4-6). More importantly, when Serine 183 alone was mutated to alanine, the protein showed a dramatic increase in exchange rate with kinetics similar to that of the C-terminal deletion mutant. This strongly suggests that although the threonine residue significantly affects the strength of binding, phosphorylation of Serine 183 appears to be the major site of regulation of histone H1 binding.

Section 4.5. Chapter Summary

It has previously been shown that an increase in histone H1 phosphorylation is associated with progression of cells through the cell cycle, and maximal phosphorylation occurs when the chromosomes are fully condensed during metaphase[134]. Furthermore, Staurosporine-induced dephosphorylation of histone H1 in mitotic cells correlated with the decondensation of the chromosomes. Although studies with phosphatase inhibitors showed that it is possible to induce chromosome condensation in the absence of histone H1 phosphorylation, this condensation is accompanied by non-physiological increases in the phosphorylation of H2A and H3, and the cells are no longer viable[158-161]. Physical studies with reconstituted chromatin showed that these phosphorylations destabilize chromatin folding[162]. Furthermore, a direct comparison between histone H1 with different levels of phosphorylation showed that this modification does not influence its binding to mononucleosomes, but increased phosphorylation levels reduced the

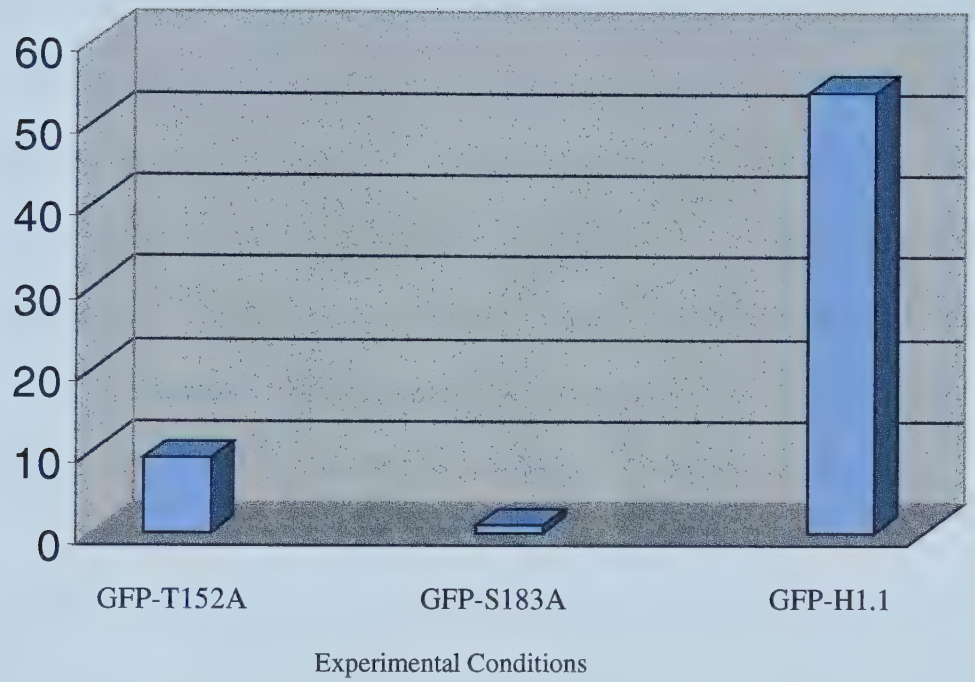

Figure 19. *$t_{½}$s for Threonine 152 and Serine 183 mutants of GFP-H1.1 compared to normal GFP-H1.1.* Quantitative analysis of FRAP experiments of SK-N-SH cells stably transfected with either GFP-T152A (mutation of Threonine 152 to alanine) or GFP-S183A (Serine 183 mutated to alanine) . $t_{½}$s are calculated from 24 GFP-T152A nuclei, 21 GFP-S183A nuclei and 51 GFP-H1.1 nuclei. Numerical values are reported in Table 7.

Table 4-6. *Time for 50% recovery and per cent mobile population of H1.1 mutants in which either Threonine 152 or Serine 183 are substituted to alanine.*

Treatment	$t_{1/2}$ (s)	Mobile Population (%)
GFP-T152A	9.0 ± 4.1* (n=24)	101.2 ± 3.7*
GFP-S183A	0.9 ± 0.43* (n= 21)	100.9 ± 4.2*
GFP-H1.1	53.9 ± 9.4 (n=51)	89.6 ± 4.7

Values reported are averages from FRAP experiments of stably transfected SK-N-SH and A549 cells. The number of nuclei (n) examined in each case is reported.
*Probability that the means are equal is less than 0.005 (compared to the full-length protein)

ability of chromatin to aggregate[162]. The available data suggest that the phosphorylation of histone H1 could relieve constraints within the chromatin and allow for structural reorganization during chromosome condensation. Interestingly, Chadee et al, have demonstrated that interphase phosphorylation of histone H1 is increased in some *ras* transformed cell lines as well as in other transformed cell lines from which the oncogene is downstream of *ras*[95,96]. Together, this may suggest that interphase hyperphosphorylation of histone H1 in transformed cell lines results in a loss of the histone H1-repressive chromatin folding and thus allows for aberrant overexpression of a large number of genes that is characteristic of cancer.

In addition, recent studies with *Tetrahymena* showed that phosphorylation of histone H1 functions to change the overall charge of a domain, causing the removal of the linker histone from chromatin and affecting specific gene expression[100,163]. This effect of phosphorylation on gene transcription was similar to that seen when histone H1 was deleted[100,163]. In support of this, we find that the C-terminal tail of histone H1.1, which contains the two phosphorylation sites, is a major regulator of its stabilization. When the full-length H1.1-GFP was reconstructed with the GFP tagged to the N-terminus, there was a dramatic decrease in the exchange rate of the protein, suggesting that it is more stably bound. We believe this results because the C-terminus binding was less inhibited when not tagged to the GFP and therefore provided a more stable interaction with chromatin. In addition, when the N-terminus of histone H1.1 was deleted, although there was an increase in the exchange rate, the effect of Staurosporine on the protein was the same as that for the full-length. This

indicates that phosphorylation of the N-terminus, or a protein regulating it, does not play a major role in stabilizing histone H1 binding. However, when the C-terminus was deleted, there was a dramatic decrease in the time required for 50% of the protein to redistribute (from 18.7 seconds to less than one second). In addition, there were minimal effects seen from Staurosporine treatment. Together, this strongly suggests that it is phosphorylation of the C-terminus of histone H1.1, and not that of another protein, that is regulating its binding and exchange.

To further characterize histone H1 binding, we investigated the dynamics of histone H1.2 binding and exchange. We found that like histone H1.1, histone H1.2 showed rapid mobility within nuclear chromatin of living cells and was similarly affected by treatment with kinase inhibitors, suggesting that binding of H1.2 is also modulated by phosphorylation.

In addition, we performed FRAP experiments on GFP fusion proteins in which either Threonine 152 or Serine 183 of histone H1.1 were substituted to alanine (mimicking the non-phosphorylated state) and observed a dramatic increase in the exchange rate of histone H1.1. Interestingly, we show that when the threonine is substituted there is a two fold increase in the exchange of the protein; however, the serine mutant behaves with kinetics similar to that of the C-terminal deletion mutant. This suggests a novel function for histone H1 phosphorylation in that the specific phosphorylated serine appears critical for stable binding to chromatin.

Chapter 5: Discussion

It is well known that histone H1 binds to the repeating structural unit of chromatin, the nucleosome and stabilizes its higher-order folding from a 10 nm to 30 nm fiber[82,87,88,103]. Histone H1 has been shown to act as both a general repressor of transcription, as well as a specific activator, most likely through this ability to condense, or better, decondense chromatin[3,4,105,106]. However, studies of *Tetrahymena* have demonstrated that although histone H1 stabilizes the folding of chromatin into a higher-order structure, it is not essential for cell viability[155]. In fact, there is little known about the exact binding and function of histone H1 *in vivo* as studies of its dynamics, until recently, have been limited to *in vitro* experimentation.

The discovery of Green Fluorescent Protein (GFP) has made it possible to look at the dynamics of cellular proteins in live cells. In particular, GFP-tagged nuclear proteins have been demonstrated to be a reliable method for measuring the mobility of these proteins with the nucleus in living cells[145,153,154,164]. Gunjan et al., were the first to characterize the dynamics of a histone H1-GFP fusion protein[144]. Through micrococcal nuclease experiments and nucleoprotein gel analysis, they show that nucleosomal binding of the fusion protein appears identical to that of the native histone H1 histone. Furthermore, Misteli et al., have reported the binding characteristics of two mouse histone H1 variants in live mouse cells using histone

H1-GFP fusion proteins[154]. The cells they use differ from human cells in that they have larger, more distinct, heterochromatic regions which may explain differences in our results. Based on fluorescence recovery after photobleaching (FRAP) experiments, Misteli and colleagues found that histone H1 exchange in mouse cells is also rapid and incomplete with a fraction of the population being immobile[154]. Upon quantitation of the immobile fraction, they find that euchromatin has less than 10% immobile (similar to our results), while heterochromatin showed approximately 25% immobile. They also report a significant increase in the exchange rate (or 'looser' binding) upon treatment with the histone deacetylase inhibitor trichostatin A (TSA). As we measured similar exchange kinetics in different regions of chromatin (condensed verses uncondensed) and observed no measurable effects upon treatment with TSA (data not shown), we believe these differences may be due to the properties of our respective cell lines. However, and more importantly, similar to our studies, they showed by HPLC profiling that the H1-GFP fusion protein comprises less than 5% of the total histone H1, that the fusion protein remains bound to DNA throughout the cell cycle and has similar salt elution properties as the native histone H1s. In addition, they further demonstrate that the fusion proteins were released from chromatin upon micrococcal nuclease digestion with kinetics identical to that of the endogenous mouse histones. Together, these studies provide strong support for the use of GFP-tagged histones in living systems.

We chose to use a H1.1-GFP fusion protein with time-lapse live cell imaging in order to follow the movement of human histone H1 in real time and characterize how

exchange was regulated. We have shown that H1.1-GFP binds to chromatin in a manner similar to native histone H1. The GFP also did not appear to affect the ability of histone H1.1 to bind nucleosomes and hence could be used as a reliable estimate for kinetic analysis of its exchange in living cells.

Contrary to what we have shown in living cells, *in vitro* experiments on histone H1 exchange show that the histone is allowed to freely move both on and off of chromatin[136-141]. These studies also demonstrate that exchange is restricted to movement within certain types of chromatin[140]. The two populations of chromatin that result from its isolation are aggregation-resistant and aggregation-prone. Highly acetylated chromatin is soluble in physiological ionic strength buffers (aggregation-resistant) and is thus more closely associated with euchromatin. Therefore, the aggregation-prone chromatin population is generally compared to heterochromatin. Work done by Jin and Cole, show that histone H1 can exchange, *in vitro*, within a population of fragments generated from condensed chromatin or a population of fragments from uncondensed chromatin but not between the two populations[141]. They suggest that since fragments from each chromatin population have different biochemical properties, and therefore do not cross-react, histone H1 exchange is restricted because direct fiber-fiber interaction is necessary for the histone to "jump" from one binding site to another[141]. *In vitro* studies of isolated nuclei also show that histone H1 associated with chromatin within the nucleus does not exchange with chromatin outside of the nucleus, even though isolated histone H1 is known to be freely diffusible across the nuclear membrane. Our experiments similarly show that

exchange of H1 histones in live cells is restricted to the nucleus and does not cross the nuclear membrane(data not shown), similar to *in vitro* results. These results further suggest that direct fiber-to-fiber contact may be needed for histone H1 to exchange both *in vitro* and *in vivo*. Interestingly however, we have shown that in live cells, although histone H1 exchange is restricted to the nucleus, it is not restricted to different classes of chromatin. In addition, we have been the first to demonstrate that the mobility of this linker histone is not restricted to chromatin fragments that are in direct contact but rather histone H1 moves freely through the nucleoplasm as a soluble intermediate. Evidence to support this and as presented in this thesis is summarized below:

1) In live, bi-nucleated cells, when one entire nucleus was bleached and half of the other, histone H1 exchange occurred rapidly and completely in the half bleached nucleus; however, there was no recovery of intensity in the fully bleached nucleus, showing that histone H1 does not exchange across the nuclear membrane, similar to *in vitro* results (data not shown).

2) Complete recovery of histone H1 is seen in both highly condensed (comparative to heterochromatin) and less condensed (euchromatic) regions with similar kinetics, demonstrating that exchange is not restricted to different chromatin classes.

3) Photobleaching experiments of lagging fluorescent chromosomes (found during metaphase and physically separated from the larger chromosomal mass), as well as isolated apoptotic chromatin bodies (found in spontaneously arising apoptotic cells), show complete recovery of H1.-GFP fluorescence in these physically isolated chromatin bodies with kinetics similar to interphase mobility, confirming that histone H1 exchange is not restricted to chromatin fragments that are in direct contact but rather it moves through the nucleoplasm as a soluble intermediate.

A rapidly growing field of research into nuclear protein mobility has demonstrated that certain proteins and sub-nuclear structures are able to move about the nucleus by Brownian motion[165], whereas other proteins appear relatively immobile. Studies of the movement of proteins through nuclear space in live cells provides more insight into the possible mechanism by which a protein acts, as compared to *in vitro* measurements of affinity constants. Because binding of histone H1 has been shown to stably repress a large number of genes through its higher-order folding of chromatin, and because of the strict requirement of histone H1 nucleosomal binding for chromatin folding, it has long been thought that this histone is stably bound. Interestingly, in our studies of histone H1 dynamics within living cells, we have shown that histone H1 is not stably bound to DNA but rather exchanges rapidly on and off of nuclear chromatin. We have further demonstrated that histone H1 is not allowed to freely exchange, but rather its' highly transient binding requires certain

phosphorylation events for exchange within the nucleus of living cells. Evidence supporting this model and presented in this thesis are summarized as follows:

1) The $t_{½}$ of histone H1.1-GFP and H1.2-GFP are approximately 18.7 and 16.2 seconds, respectively; whereas the $t_{½}$ for unbound, empty vector-GFP is less than one second (representing free diffusion) and that for another nucleosomal histone, H2B, which is of similar size to H1.1 is greater than 30 minutes – demonstrating the rapid mobility of histone H1.

2) There is no exchange of H1.1-GFP in necrotic cells; however, brief treatment of cells with ATP-depleting agents minimally affects the exchange rate of H1.1-GFP, and prolonged treatment with either ATP-depleting agents or mitochondrial poisons (Sodium Azide) results in a dramatic decrease in the exchange rate of the protein, as well as a significant increase in the immobile population of the protein – suggesting that histone H1 exchange does not depend on the availability of energy *per se*, but rather on phosphorylation of either itself or another protein regulating its binding.

3) Treatment of cells stably expressing H1.1-GFP (with or without its N-terminus) with the general kinase inhibitor, Staurosporine, led to an approximate doubling of the exchange rate of histone H1, providing

stronger evidence that its binding and mobility are regulated by phosphorylation.

Although we had demonstrated that phosphorylation regulates histone H1 binding and exchange, it was critical to determine whether it was phosphorylation of the histone itself, or that of another protein involved in its binding, that was the major regulator in stabilizing its binding. In support of the former, it has been well documented that histone H1 phosphorylation is transient throughout the cell cycle[134]. During mitosis, it becomes hyperphosphorylated to allow for chromatin condensation and subsequent chromosome formation. For this reason, it is not surprising that phosphorylation of the histone may play a role in its ability to bind and release chromatin. Our initial experiments characterized histone H1 binding with an H1-GFP fusion protein in which the GFP was tagged to the C-terminus. Although we show that histone H1.1 nucleosomal binding does not appear to be affected by the GFP tag, when the GFP is placed on the N-terminus the strength of binding *in vivo* is significantly increased. This suggests that the C-terminus (with the only two phosphorylation sites) is critical for histone H1 binding and that the GFP tag weakens the interaction between H1.1-GFP and DNA. However, it is important to note that the GFP-H1.1 fusion protein is similarly affected by treatment with kinase inhibitors as H1.1-GFP, suggesting that the GFP tag does not interfere with normal histone H1 phosphorylation. In addition, we have demonstrated that the N-terminals tail of histones H1.1 and H1.2 play a significant role in stabilizing histone H1 binding. Furthermore, we show that when the C-terminus of histone H1 is

deleted, approximately 100% of the protein becomes mobile (compared to 92% for the full-length protein). In addition, the exchange rate of the protein is almost forty times faster than full-length histone H1, clearly demonstrating that the C-terminus plays a major role in stabilizing binding. Importantly, we have identified the C-terminal tail of histone H1 to be a major regulator in its binding and exchange and have further demonstrated that phosphorylation of the C-terminus of histone H1 itself, and not that of another protein, appears critical for its mobility. Evidence supporting this is summarized as follows:

1) Tagging the GFP to the N-terminal tail of histone H1.1 led to a decrease in the exchange rate of the protein (a $t_{½}$ of 54 seconds for GFP-H1.1 versus 19 H1.1-GFP), indicating that the uninhibited C-terminus of GFP-H1.1 is more stably bound, suggesting that the C-terminal tail plays a major role in stabilizing histone H1 binding.

2) Deletion of the N-terminal tail of histone H1.1 and (with no phosphorylation sites) showed a significant increase in exchange rate; however, was similarly affected by treatment with kinase inhibitors compared to that of the full-length protein, suggesting that although the N-terminus is involved in binding, it does not appear to be affected by the phosphorylation status of the cell.

3) Deletion of the C-terminus of histone H1.1 (with the 2 possible phosphorylation sites) led to a dramatic increase in mobility of the protein (similar to that of free diffusion) and was not significantly affected by treatment with Staurosporine, providing strong evidence that it is phosphorylation of the C-terminal tail of histone H1 itself, and not that of another protein, that is regulating its binding and exchange.

Although these results are consistent with our hypothesis of a role for phosphorylation and H1, preliminary data on early G1 cells, where steady-state phosphorylation is minimal, contradict the possibility that histone H1 is the target for the phosphorylation that mediates its exchange. These early G1 cells do not differ significantly in their exchange rate from the total interphase population. However, more investigation into the levels of histone H1 phosphorylation in these cells is necessary. Other preliminary experiments we've initiated have further characterized the role of the histone H1.1 C-terminal tail in regulating its binding and mobility and thus also support our revised hypothesis. Site-directed mutagenesis was performed to substitute either of the two phosphorylation sites in the C-terminus to alanine. FRAP experiments of GFP fusion proteins in which either Threonine 152 or Serine 183 of histone H1.1 were substituted to alanine show dramatic changes in the exchange rate of the histone. Surprisingly, we show that when the threonine is substituted there is a two fold increase in the exchange of the protein; however, the serine mutant behaves with kinetics similar to that of the C-terminal deletion mutant. Although more

investigation is required into the specific C-terminal phosphorylation sites of histones H1.1 and H1.2, as well as the other histone H1 variants, these early results provide strong evidence that mutation of Serine 183 of histone H1.1 to the non-phosphorylated state acts to completely destabilize the histones binding and displace the histone from chromatin. This introduces a new role for histone H1 phosphorylation in that it appears, from our results, that a specific phosphorylated residue is necessary for stable binding of the protein, contrary to the widely accepted belief that phosphorylation acts solely to weaken the protein-DNA interaction. Future directions for these experiments will involve studying mutants in which one or all of the histone H1 phosphorylation sites are mutated to glutamic acid (mimicking the phosphorylated state) as well as a combination of alanine and glutamine substitutions to measure the relative contributions of each charged residue. Futhermore, the experiments will be extended to include additional human histone H1 variants.

6. BIBLIOGRAPHY

1. Luger, K. & Richmond, T.J. The histone tails of the nucleosome. *Curr Opin Genet Dev* **8**, 140-6. (1998).
2. Kornberg, R.D. & Lorch, Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**, 285-94. (1999).
3. Wolffe, A.P., Khochbin, S. & Dimitrov, S. What do linker histones do in chromatin? *Bioessays* **19**, 249-55. (1997).
4. Thomas, J.O. Histone H1: location and role. *Curr Opin Cell Biol* **11**, 312-7. (1999).
5. Davie, J.R. & Spencer, V.A. Control of histone modifications. *J Cell Biochem* **Suppl**, 141-8. (1999).
6. Strahl, B.D. & Allis, C.D. The language of covalent histone modifications. *Nature* **403**, 41-5. (2000).
7. Jenuwein, T. & Allis, C.D. Translating the histone code. *Science* **293**, 1074-80. (2001).
8. Reuter, G., Werner, W. & Hoffmann, H.J. Mutants affecting position-effect heterochromatinization in Drosophila melanogaster. *Chromosoma* **85**, 539-51 (1982).
9. Grigliatti, T. Position-effect variegation--an assay for nonhistone chromosomal proteins and chromatin assembly and modifying factors. *Methods Cell Biol* **35**, 587-627 (1991).

10. Lu, B.Y. & Eissenberg, J.C. Time out: developmental regulation of heterochromatic silencing in Drosophila. *Cell Mol Life Sci* **54**, 50-9. (1998).

11. Kornberg, R.D. & Lorch, Y. Chromatin-modifying and -remodeling complexes. *Curr Opin Genet Dev* **9**, 148-51. (1999).

12. Wolffe, A.P. & Hayes, J.J. Chromatin disruption and modification. *Nucleic Acids Res* **27**, 711-20. (1999).

13. Wallrath, L.L. Unfolding the mysteries of heterochromatin. *Curr Opin Genet Dev* **8**, 147-53. (1998).

14. Heitz, E. Das Heterochromatin der Moose. *Jhrb. Wiss. Botanik* **69**, 762-818 (1928).

15. Singh, P.B. *et al.* A sequence motif found in a Drosophila heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res* **19**, 789-94. (1991).

16. Elgin, S.C. Heterochromatin and gene regulation in Drosophila. *Curr Opin Genet Dev* **6**, 193-202. (1996).

17. Henikoff, S. Heterochromatin function in complex genomes. *Biochim Biophys Acta* **1470**, O1-8. (2000).

18. Gottschling, D.E., Aparicio, O.M., Billington, B.L. & Zakian, V.A. Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. *Cell* **63**, 751-62. (1990).

19. Cockell, M. *et al.* The carboxy termini of Sir4 and Rap1 affect Sir3 localization: evidence for a multicomponent complex required for yeast telomeric silencing. *J Cell Biol* **129**, 909-24. (1995).

20. Maillet, L. *et al.* Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer- mediated repression. *Genes Dev* **10**, 1796-811. (1996).

21. Allshire, R.C. Centromeres, checkpoints and chromatid cohesion. *Curr Opin Genet Dev* **7**, 264-73. (1997).

22. Henikoff, S., Jackson, J.M. & Talbert, P.B. Distance and pairing effects on the brownDominant heterochromatic element in Drosophila. *Genetics* **140**, 1007-17. (1995).

23. Weiler, K.S. & Wakimoto, B.T. Heterochromatin and gene expression in Drosophila. *Annu Rev Genet* **29**, 577-605 (1995).

24. Dernburg, A.F. *et al.* Perturbation of nuclear architecture by long-distance chromosome interactions. *Cell* **85**, 745-59. (1996).

25. Brown, K.E. *et al.* Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* **91**, 845-54. (1997).

26. Singh, J. & Klar, A.J. Active genes in budding yeast display enhanced in vivo accessibility to foreign DNA methylases: a novel in vivo probe for chromatin structure of yeast. *Genes Dev* **6**, 186-96. (1992).

27. Grunstein, M. Yeast heterochromatin: regulation of its assembly and inheritance by histones. *Cell* **93**, 325-8. (1998).

28. Mottus, R., Reeves, R. & Grigliatti, T.A. Butyrate suppression of position-effect variegation in Drosophila melanogaster. *Mol Gen Genet* **178**, 465-9 (1980).

29. Wustmann, G., Szidonya, J., Taubert, H. & Reuter, G. The genetics of position-effect variegation modifying loci in Drosophila melanogaster. *Mol Gen Genet* **217**, 520-7. (1989).

30. James, T.C. & Elgin, S.C. Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. *Mol Cell Biol* **6**, 3862-72. (1986).

31. James, T.C. *et al.* Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. *Eur J Cell Biol* **50**, 170-80. (1989).

32. Eissenberg, J.C. *et al.* Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **87**, 9923-7. (1990).

33. Eissenberg, J.C., Morris, G.D., Reuter, G. & Hartnett, T. The heterochromatin-associated protein HP-1 is an essential protein in Drosophila with dosage-dependent effects on position-effect variegation. *Genetics* **131**, 345-52. (1992).

34. Lorentz, A., Heim, L. & Schmidt, H. The switching gene swi6 affects recombination and gene expression in the mating-type region of Schizosaccharomyces pombe. *Mol Gen Genet* **233**, 436-42. (1992).

35. Festenstein, R. *et al.* Heterochromatin protein 1 modifies mammalian PEV in a dose- and chromosomal-context-dependent manner. *Nat Genet* **23**, 457-61. (1999).

36. Ma, J., Hwang, K.K., Worman, H.J., Courvalin, J.C. & Eissenberg, J.C. Expression and functional analysis of three isoforms of human heterochromatin-associated protein HP1 in Drosophila. *Chromosoma* **109**, 536-44. (2001).

37. Aasland, R. & Stewart, A.F. The chromo shadow domain, a second chromo domain in heterochromatin- binding protein 1, HP1. *Nucleic Acids Res* **23**, 3168-74. (1995).

38. Koonin, E.V., Zhou, S. & Lucchesi, J.C. The chromo superfamily: new members, duplication of the chromo domain and possible role in delivering transcription regulators to chromatin. *Nucleic Acids Res* **23**, 4229-33. (1995).

39. Ball, L.J. *et al.* Structure of the chromatin binding (chromo) domain from mouse modifier protein 1. *Embo J* **16**, 2473-81. (1997).

40. Yamaguchi, K., Hidema, S. & Mizuno, S. Chicken chromobox proteins: cDNA cloning of CHCB1, -2, -3 and their relation to W-heterochromatin. *Exp Cell Res* **242**, 303-14. (1998).

41. Saunders, W.S. *et al.* Molecular cloning of a human homologue of Drosophila heterochromatin protein HP1 using anti-centromere autoantibodies with anti-chromo specificity. *J Cell Sci* **104**, 573-82. (1993).

42. Ye, Q. & Worman, H.J. Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to Drosophila HP1. *J Biol Chem* **271**, 14653-6. (1996).

43. Ye, Q., Callebaut, I., Pezhman, A., Courvalin, J.C. & Worman, H.J. Domain-specific interactions of human HP1-type chromodomain proteins and inner nuclear membrane protein LBR. *J Biol Chem* **272**, 14983-9. (1997).

44. Nielsen, A.L. *et al.* Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. *Embo J* **18**, 6385-95. (1999).

45. Minc, E., Courvalin, J.C. & Buendia, B. HP1gamma associates with euchromatin and heterochromatin in mammalian nuclei and chromosomes. *Cytogenet Cell Genet* **90**, 279-84 (2000).

46. Furuta, K. *et al.* Heterochromatin protein HP1Hsbeta (p25beta) and its localization with centromeres in mitosis. *Chromosoma* **106**, 11-9. (1997).

47. Minc, E., Allory, Y., Worman, H.J., Courvalin, J.C. & Buendia, B. Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* **108**, 220-34. (1999).

48. Le Douarin, B. *et al.* A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. *Embo J* **15**, 6701-15. (1996).

49. Zhao, T., Heyduk, T., Allis, C.D. & Eissenberg, J.C. Heterochromatin protein 1 binds to nucleosomes and DNA in vitro. *J Biol Chem* **275**, 28332-8. (2000).

50. Eissenberg, J.C. & Elgin, S.C. The HP1 protein family: getting a grip on chromatin. *Curr Opin Genet Dev* **10**, 204-10. (2000).

51. Jones, D.O., Cowell, I.G. & Singh, P.B. Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays* **22**, 124-37. (2000).

52. Paro, R. & Hogness, D.S. The Polycomb protein shares a homologous domain with a heterochromatin- associated protein of Drosophila. *Proc Natl Acad Sci U S A* **88**, 263-7. (1991).

53. Cleard, F., Delattre, M. & Spierer, P. SU(VAR)3-7, a Drosophila heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. *Embo J* **16**, 5280-8. (1997).

54. Seeler, J.S., Marchio, A., Sitterlin, D., Transy, C. & Dejean, A. Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc Natl Acad Sci U S A* **95**, 7316-21. (1998).

55. Huang, D.W. *et al.* Distinct cytoplasmic and nuclear fractions of Drosophila heterochromatin protein 1: their phosphorylation levels and associations with origin recognition complex proteins. *J Cell Biol* **142**, 307-18. (1998).

56. Pak, D.T. *et al.* Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91**, 311-23. (1997).

57. Ekwall, K. *et al.* The chromodomain protein Swi6: a key component at fission yeast centromeres. *Science* **269**, 1429-31. (1995).

58. Smith, S. & Stillman, B. Stepwise assembly of chromatin during DNA replication in vitro. *Embo J* **10**, 971-80. (1991).

59. Kaufman, P.D., Kobayashi, R., Kessler, N. & Stillman, B. The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication. *Cell* **81**, 1105-14. (1995).

60. Krude, T. Chromatin assembly factor 1 (CAF-1) colocalizes with replication foci in HeLa cell nuclei. *Exp Cell Res* **220**, 304-11. (1995).

61. Enomoto, S., McCune-Zierath, P.D., Gerami-Nejad, M., Sanders, M.A. & Berman, J. RLF2, a subunit of yeast chromatin assembly factor-I, is required for telomeric chromatin function in vivo. *Genes Dev* **11**, 358-70. (1997).

62. Kaufman, P.D., Kobayashi, R. & Stillman, B. Ultraviolet radiation sensitivity and reduction of telomeric silencing in Saccharomyces cerevisiae cells lacking chromatin assembly factor-I. *Genes Dev* **11**, 345-57. (1997).

63. Murzina, N., Verreault, A., Laue, E. & Stillman, B. Heterochromatin dynamics in mouse cells: interaction between chromatin assembly factor 1 and HP1 proteins. *Mol Cell* **4**, 529-40. (1999).

64. Bradbury, E.M. Reversible histone modifications and the chromosome cell cycle. *Bioessays* **14**, 9-16. (1992).

65. Kuo, M.H. & Allis, C.D. Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* **20**, 615-26. (1998).

66. Turner, B.M. Histone acetylation and an epigenetic code. *Bioessays* **22**, 836-45. (2000).

67. Brownell, J.E. & Allis, C.D. Special HATs for special occasions: linking histone acetylation to chromatin assembly and gene activation. *Curr Opin Genet Dev* **6**, 176-84. (1996).

68. Taunton, J., Hassig, C.A. & Schreiber, S.L. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* **272**, 408-11. (1996).

69. Hsu, J.Y. *et al.* Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. *Cell* **102**, 279-91. (2000).

70. Rea, S. *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**, 593-9. (2000).

71. Tschiersch, B. *et al.* The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. *Embo J* **13**, 3822-31. (1994).

72. Aagaard, L. *et al.* Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3- 9 encode centromere-associated proteins which complex with the heterochromatin component M31. *Embo J* **18**, 1923-38. (1999).

73. Aagaard, L., Schmid, M., Warburton, P. & Jenuwein, T. Mitotic phosphorylation of SUV39H1, a novel component of active centromeres, coincides with transient accumulation at mammalian centromeres. *J Cell Sci* **113**, 817-29. (2000).

74. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**, 116-20. (2001).

75. Hendzel, M.J. *et al.* Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-60. (1997).

76. Nielsen, A.L. *et al.* Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. *Mol Cell* **7**, 729-39. (2001).

77. Allan, J., Hartman, P.G., Crane-Robinson, C. & Aviles, F.X. The structure of histone H1 and its location in chromatin. *Nature* **288**, 675-9. (1980).

78. Staynov, D.Z. & Crane-Robinson, C. Footprinting of linker histones H5 and H1 on the nucleosome. *Embo J* **7**, 3685-91. (1988).

79. Hartman, P.G., Chapman, G.E., Moss, T. & Bradbury, E.M. Studies on the role and mode of operation of the very-lysine-rich histone H1 in eukaryote chromatin. The three structural regions of the histone H1 molecule. *Eur J Biochem* **77**, 45-51. (1977).

80. Allan, J., Mitchell, T., Harborne, N., Bohm, L. & Crane-Robinson, C. Roles of H1 domains in determining higher order chromatin structure and H1 location. *J Mol Biol* **187**, 591-601. (1986).

81. Goytisolo, F.A. *et al.* Identification of two DNA-binding sites on the globular domain of histone H5. *Embo J* **15**, 3421-9. (1996).

82. Ramakrishnan, V. Histone structure and the organization of the nucleosome. *Annu Rev Biophys Biomol Struct* **26**, 83-112 (1997).

83. Czupryn, M. & Toczko, K. Lack of nucleosomal structure in a DNase-I-solubilized transcriptionally active chromatin fraction of Physarum polycephalum. *Eur J Biochem* **147**, 575-80. (1985).

84. Hayes, J.J. Site-directed cleavage of DNA by a linker histone--Fe(II) EDTA conjugate: localization of a globular domain binding site within a nucleosome. *Biochemistry* **35**, 11931-7. (1996).

85. Pruss, D. *et al.* An asymmetric model for the nucleosome: a binding site for linker histones inside the DNA gyres. *Science* **274**, 614-7. (1996).

86. Vignali, M. & Workman, J.L. Location and function of linker histones. *Nat Struct Biol* **5**, 1025-8. (1998).

87. Widom, J. Structure, dynamics, and function of chromatin in vitro. *Annu Rev Biophys Biomol Struct* **27**, 285-327 (1998).

88. Widom, J. Chromatin structure: linking structure to function with histone H1. *Curr Biol* **8**, R788-91. (1998).

89. Breneman, J.W., Yau, P., Teplitz, R.L. & Bradbury, E.M. A light microscope study of linker histone distribution in rat metaphase chromosomes and interphase nuclei. *Experimental Cell Research* **206**, 16-26 (1993).

90. Parseghian, M.H. *et al.* Fractionation of human H1 subtypes and characterization of a subtype-specific antibody exhibiting non-uniform nuclear staining. *Chromosome Res* **1**, 127-139 (1993).

91. Parseghian, M.H., Harris, D.A., Rishwain, D.R. & Hamkalo, B.A. Characterization of a set of antibodies specific for three human histone H1 subtypes. *Embo J* **103**, 198-208. (1994).

92. Clark, D.J., Hill, C.S., Martin, S.R. & Thomas, J.O. Alpha-helix in the carboxy-terminal domains of histones H1 and H5. *Embo J* **7**, 69-75. (1988).

93. Smith, R.C., Dworkin-Rastl, E. & Dworkin, M.B. Expression of a histone H1-like protein is restricted to early Xenopus development. *Genes Dev* **2**, 1284-95. (1988).

94. Albig, W., Kardalinou, E., Drabent, E., Zimmer, A. & Doenecke, D. Isolation and characterization of two human H1 genes within clusters of core histone genes. *Embo J* **10**, 940-948 (1991).

95. Chadee, D.N. *et al.* Increased phosphorylation of histone H1 in mouse fibroblasts transformed with oncogenes or constitutively active mitogen-activated protein kinase kinase. *J Biol Chem* **270**, 20098-105. (1995).

96. Chadee, D.N., Allis, C.D., Wright, J.A. & Davie, J.R. Histone H1b phosphorylation is dependent upon ongoing transcription and replication in normal and ras-transformed mouse fibroblasts. *J Biol Chem* **272**, 8113-6. (1997).

97. Brown, D.T., Alexander, B.T. & Sittman, D.B. Differential effect of H1 variant overexpression on cell cycle progression and gene expression. *Nucleic Acids Research* **24**, 486-493 (1996).

98. Takami, Y. & Nakayama, T. A single copy of linker H1 genes is enough for proliferation of the DT40 chicken B cell line, and linker H1 variants participate in regulation of gene expression. *Genes Cell* **2**, 711-723 (1997).

99. Ohsumi, K., Katagiri, C. & Kishimoto, T. Chromosome condensation in Xenopus mitotic extracts without histone H1. *Science* **262**, 2033-5. (1993).

100. Dou, Y., Mizzen, C.A., Abrams, M., Allis, C.D. & Gorovsky, M.A. Phosphorylation of linker histone H1 regulates gene expression in vivo by mimicking H1 removal. *Mol Cell* **4**, 641-7. (1999).

101. Bradbury, E.M. & Baldwin, J.P. Neutron scatter studies of chromatin structure. *Supramolecular Structure and Function* , 14533-6. (1986).

102. Van Holde, K.E. Chromatin. *Springer-Verlag, New York, Heidelberg* (1988).

103. Widom, J. Toward a unified model of chromatin folding. *Annu Rev Biophys Biophys Chem* **18**, 365-95 (1989).

104. Yoshikawa, Y., Velichko, Y.S., Ichiba, Y. & Yoshikawa, K. Self-assembled pearling structure of long duplex DNA with histone H1. *Eur J Biochem* **268**, 2593-9. (2001).

105. Zlatanova, J. & Van Holde, K. Histone H1 and transcription: still an enigma? *J Cell Sci* **103**, 889-95. (1992).

106. Shen, X. & Gorovsky, M.A. Linker histone H1 regulates specific gene expression but not global transcription in vivo. *Cell* **86**, 475-83. (1996).

107. Archer, T.K., Cordingley, M.G., Wolford, R.G. & Hager, G.L. Transcription factor access is mediated by accurately positioned nucleosomes on the mouse mammary tumor virus promoter. *Mol Cell Biol* **11**, 688-98. (1991).

108. Workman, J.L. & Kingston, R.E. Nucleosome core displacement in vitro via a metastable transcription factor-nucleosome complex. *Science* **258**, 1780-4. (1992).

109. Herrera, J.E., West, K.L., Schiltz, R.L., Nakatani, Y. & Bustin, M. Histone H1 is a specific repressor of core histone acetylation in chromatin. *Mol Cell Biol* **20**, 523-9. (2000).

110. Turner, B.M. & O'Neill, L.P. Histone acetylation in chromatin and chromosomes. *Semin Cell Biol* **6**, 229-36. (1995).

111. Struhl, K. Histone acetylation and transcriptional regulatory mechanisms. *Genes Dev* **12**, 599-606. (1998).

112. Hayes, J.J. & Wolffe, A.P. The interaction of transcription factors with nucleosomal DNA. *Bioessays* **14**, 597-603. (1992).

113. Adams, C.C. & Workman, J.L. Nucleosome displacement in transcription. *Cell* **72**, 305-8. (1993).

114. Laybourn, P.J. & Kadonaga, J.T. Role of nucleosomal cores and histone H1 in regulation of transcription by RNA polymerase II. *Science* **254**, 238-45. (1991).

115. Hansen, J.C. & Wolffe, A.P. A role for histones H2A/H2B in chromatin folding and transcriptional repression. *Proc Natl Acad Sci U S A* **91**, 2339-43. (1994).

116. Cary, P.D., Hines, M.L., Bradbury, E.M., Smith, B.J. & Johns, E.W. Conformation studies of histone H1(0) in comparison with histones H1 and H5. *Eur J Biochem* **120**, 371-7. (1981).

117. Bouvet, P., Dimitrov, S. & Wolffe, A.P. Specific regulation of Xenopus chromosomal 5S rRNA gene transcription in vivo by histone H1. *Genes Dev* **8**, 1147-59. (1994).

118. Howe, L. & Ausio, J. Nucleosome translational position, not histone acetylation, determines TFIIIA binding to nucleosomal Xenopus laevis 5S rRNA genes. *Mol Cell Biol* **18**, 1156-62. (1998).

119. Sera, T. & Wolffe, A.P. Role of histone H1 as an architectural determinant of chromatin structure and as a specific repressor of transcription on Xenopus oocyte 5S rRNA genes. *Mol Cell Biol* **18**, 3668-80. (1998).

120. Howe, L., Itoh, T., Katagiri, C. & Ausio, J. Histone H1 binding does not inhibit transcription of nucleosomal Xenopus laevis somatic 5S rRNA templates. *Biochemistry* **37**, 7077-82. (1998).

121. Howe, L., Ranalli, T.A., Allis, C.D. & Ausio, J. Transcriptionally active Xenopus laevis somatic 5 S ribosomal RNA genes are packaged with hyperacetylated histone H4, whereas transcriptionally silent oocyte genes are not. *J Biol Chem* **273**, 20693-6. (1998).

122. Juan, L.J., Utley, R.T., Vignali, M., Bohm, L. & Workman, J.L. H1-mediated repression of transcription factor binding to a stably positioned nucleosome. *J Biol Chem* **272**, 3635-40. (1997).

123. Polach, K.J. & Widom, J. Mechanism of protein access to specific DNA sequences in chromatin: a dynamic equilibrium model for gene regulation. *J Mol Biol* **254**, 130-49. (1995).

124. Polach, K.J., Lowary, P.T. & Widom, J. Effects of core histone tail domains on the equilibrium constants for dynamic DNA site accessibility in nucleosomes. *J Mol Biol* **298**, 211-23. (2000).

125. Simpson, R.T., Whitlock, J.P., Jr., Bina-Stein, M. & Stein, A. Histone-DNA interactions in chromatin core particles. *Cold Spring Harb Symp Quant Biol* **42**, 127-36. (1978).

126. Cowman, M.K. & Fasman, G.D. Dependence of mononucleosome deoxyribonucleic acid conformation on the deoxyribonucleic acid length and H1/H5 content. Circular dichroism and thermal denaturation studies. *Biochemistry* **19**, 532-41. (1980).

127. Muyldermans, S., Lasters, I., Wyns, L. & Hamers, R. Protection of discrete DNA fragments by the complex H1-octamerhistones or H5-octamerhistones after micrococcal nuclease digestion. *Nucleic Acids Res* **9**, 3671-80. (1981).

128. Ivanchenko, M., Hassan, A., van Holde, K. & Zlatanova, J. H1 binding unwinds DNA. Evidence from topological assays. *J Biol Chem* **271**, 32580-5. (1996).

129. Davie, J.R. & Saunders, C.A. Chemical composition of nucleosomes among domains of calf thymus chromatin differing in micrococcal nuclease accessibility and solubility properties. *J Biol Chem* **256**, 12574-80. (1981).

130. Ridsdale, J.A. & Davie, J.R. Chicken erythrocyte polynucleosomes which are soluble at physiological ionic strength and contain linker histones are highly enriched in beta- globin gene sequences. *Nucleic Acids Res* **15**, 1081-96. (1987).

131. Kamakaka, R.T. & Thomas, J.O. Chromatin structure of transcriptionally competent and repressed genes. *Embo J* **9**, 3997-4006. (1990).

132. Dedon, P.C., Soults, J.A., Allis, C.D. & Gorovsky, M.A. Formaldehyde cross-linking and immunoprecipitation demonstrate developmental changes in H1 association with transcriptionally active genes. *Mol Cell Biol* **11**, 1729-33. (1991).

133. Lee, H.L. & Archer, T.K. Prolonged glucocorticoid exposure dephosphorylates histone H1 and inactivates the MMTV promoter. *Embo J* **17**, 1454-66. (1998).

134. Roth, S.Y. & Allis, C.D. Chromatin condensation: does histone H1 dephosphorylation play a role? *Trends Biochem Sci* **17**, 93-8. (1992).

135. Gurley, L.R., Valdez, J.G. & Buchanan, J.S. Characterization of the mitotic specific phosphorylation site of histone H1. Absence of a consensus sequence for the p34cdc2/cyclin B kinase. *J Biol Chem* **270**, 27653-60. (1995).

136. Caron, F. & Thomas, J.O. Exchange of histone H1 between segments of chromatin. *J Mol Biol* **146**, 513-37. (1981).

137. Louters, L. & Chalkley, R. In vitro exchange of nucleosomal histones H2a and H2b. *Biochemistry* **23**, 547-52. (1984).

138. Louters, L. & Chalkley, R. Exchange of histones H1, H2A, and H2B in vivo. *Biochemistry* **24**, 3080-5. (1985).

139. Thomas, J.O. & Rees, C. Exchange of histones H1 and H5 between chromatin fragments. A preference of H5 for higher-order structures. *Eur J Biochem* **134**, 109-15. (1983).

140. Jin, Y.J. & Cole, R.D. H1 histone exchange is limited to particular regions of chromatin that differ in aggregation properties. *J Biol Chem* **261**, 3420-7. (1986).

141. Jin, Y.J. & Cole, R.D. Exchange of H1 histone depends on aggregation of chromatin, not simply on ionic strength. *J Biol Chem* **261**, 15805-12. (1986).

142. Schwarz, P.M., Felthauser, A., Fletcher, T.M. & Hansen, J.C. Reversible oligonucleosome self-association: dependence on divalent cations and core histone tail domains. *Biochemistry* **35**, 4009-15. (1996).

143. Wu, L.H., Kuehl, L. & Rechsteiner, M. Dynamic behavior of histone H1 microinjected into HeLa cells. *J Cell Biol* **103**, 465-74. (1986).

144. Gunjan, A., Alexander, B.T., Sittman, D.B. & Brown, D.T. Effects of H1 histone variant overexpression on chromatin structure. *J Biol Chem* **274**, 37950-6. (1999).

145. Belmont, A.S. Visualizing chromosome dynamics with GFP. *Trends Cell Biol* **11**, 250-7. (2001).

146. Belmont, A.S., Dietzel, S., Nye, A.C., Strukov, Y.G. & Tumbar, T. Large-scale chromatin structure and function. *Curr Opin Cell Biol* **11**, 307-11. (1999).

147. Lamond, A.I. & Earnshaw, W.C. Structure and function in the nucleus. *Science* **280**, 547-53. (1998).

148. Kurz, A. *et al.* Active and inactive genes localize preferentially in the periphery of chromosome territories. *J Cell Biol* **135**, 1195-205. (1996).

149. Wansink, D.G., Sibon, O.C., Cremers, F.F., van Driel, R. & de Jong, L. Ultrastructural localization of active genes in nuclei of A431 cells. *J Cell Biochem* **62**, 10-8. (1996).

150. Ridsdale, J.A., Hendzel, M.J., Delcuve, G.P. & Davie, J.R. Histone acetylation alters the capacity of the H1 histones to condense transcriptionally active/competent chromatin. *J Biol Chem* **265**, 5150-6. (1990).

151. Prasher, D.C. Using GFP to see the light. *Trends Genet* **11**, 320-3. (1995).

152. Li, W., Nagaraja, S., Delcuve, G.P., Hendzel, M.J. & Davie, J.R. Effects of histone acetylation, ubiquitination and variants on nucleosome stability. *Biochem J* **296**, 737-44. (1993).

153. Kruhlak, M.J. *et al.* Reduced mobility of the alternate splicing factor (ASF) through the nucleoplasm and steady state speckle compartments. *J Cell Biol* **150**, 41-51. (2000).

154. Misteli, T., Gunjan, A., Hock, R., Bustin, M. & Brown, D.T. Dynamic binding of histone H1 to chromatin in living cells. *Nature* **408**, 877-81. (2000).

155. Shen, X., Yu, L., Weir, J.W. & Gorovsky, M.A. Linker histones are not essential and affect chromatin condensation in vivo. *Cell* **82**, 47-56. (1995).

156. Eick, S., Nicolai, M., Mumberg, D. & Doenecke, D. Human H1 histones: conserved and varied sequence elements in two H1 subtype genes. *Eur J Cell Biol* **49**, 110-5. (1989).

157. Talasz, H., Sapojnikova, N., Helliger, W., Lindner, H. & Puschendorf, B. In vitro binding of H1 histone subtypes to nucleosomal organized mouse mammary tumor virus long terminal repeat promotor. *J Biol Chem* **273**, 32236-43. (1998).

158. Roberge, M., Th'ng, J., Hamaguchi, J. & Bradbury, E.M. The topoisomerase II inhibitor VM-26 induces marked changes in histone H1 kinase activity, histones H1 and H3 phosphorylation, and chromosome condensation in G2 phase and mitotic BHK cells. *J Cell Biol* **111**, 1753-62. (1990).

159. Paulson, J.R., Ciesielski, W.A., Schram, B.R. & Mesner, P.W. Okadaic acid induces dephosphorylation of histone H1 in metaphase- arrested HeLa cells. *J Cell Sci* **107**, 267-73. (1994).

160. Guo, X.W. *et al.* Chromosome condensation induced by fostriecin does not require p34cdc2 kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation. *Embo J* **14**, 976-85. (1995).

161. Ajiro, K., Yoda, K., Utsumi, K. & Nishikawa, Y. Alteration of cell cycle-dependent histone phosphorylations by okadaic acid. Induction of mitosis-specific H3 phosphorylation and chromatin condensation in mammalian interphase cells. *J Biol Chem* **271**, 13197-201. (1996).

162. Kaplan, L.J., Bauer, R., Morrison, E., Langan, T.A. & Fasman, G.D. The structure of chromatin reconstituted with phosphorylated H1. Circular dichroism and thermal denaturation studies. *J Biol Chem* **259**, 8777-85. (1984).

163. Dou, Y. & Gorovsky, M.A. Phosphorylation of linker histone H1 regulates gene expression in vivo by creating a charge patch. *Embo J* **6**, 225-231 (2000).

164. Lever, M.A., Th'ng, J.P., Sun, X. & Hendzel, M.J. Rapid exchange of histone H1.1 on chromatin in living human cells. *Nature* **408**, 873-6. (2000).

165. Hendzel, M.J. *et al.* Compartmentalization of regulatory proteins in the cell nucleus. *J Steroid Biochem Mol Biol* **76**, 9-21. (2001).

7. APPENDICES

7.1. Appendix I

(a) H1.1	(214 aa)	SETVPPAPAASAAPEKPLAGKKAKKPAKAAAASKKKPA
(b) H1.1-ΔC	(119 aa)	SETVPPAPAASAAPEKPLAGKKAKKPAKAAAASKKKPA
(c) ΔN-H1.1	(178 aa)	PA
(d) H1.1-T152A	(214 aa)	SETVPPAPAASAAPEKPLAGKKAKKPAKAAAASKKKPA
(e) H1.1-S183A	(214 aa)	SETVPPAPAASAAPEKPLAGKKAKKPAKAAAASKKKPA

GPSVSELIVQAASSSKERGGVSLAALKKALAAAGYDVEKNNSRIKLGIKSLVSKGTLVQTKGTGASGS
GPSVSELIVQAASSSKERGGVSLAALKKALAAAGYDVEKNNSRIKLGIKSLVSKGTLVQTKGTGASGS
GPSVSELIVQAASSSKERGGVSLAALKKALAAAGYDVEKNNSRIKLGIKSLVSKGTLVQTKGTGASGS
GPSVSELIVQAASSSKERGGVSLAALKKALAAAGYDVEKNNSRIKLGIKSLVSKGTLVQTKGTGASGS
GPSVSELIVQAASSSKERGGVSLAALKKALAAAGYDVEKNNSRIKLGIKSLVSKGTLVQTKGTGASGS

FKLNKKASSVETKPGASKVATKTKATGASKKLKKATGASKKSVKTPKKAKKPAATRKSSKNPKKPKTV
FKLNKKASSVETK
FKLNKKASSVETKPGASKVATKTKATGASKKLKKATGASKKSVKTPKKAKKPAATRKSSKNPKKPKTV
FKLNKKASSVETKPGASKVATKTKATGASKKLKKATGASKKSVK**A**PKKAKKPAATRKSSKNPKKPKTV
FKLNKKASSVETKPGASKVATKTKATGASKKLKKATGASKKSVKTPKKAKKPAATRKSSKNPKKPKTV

KPKKVAKSPAKAKAVKPKAAKARVTKPKTAKPKKAAPKKK

KPKKVAKSPAKAKAVKPKAAKARVTKPKTAKPKKAAPKKK
KPKKVAKSPAKAKAVKPKAAKARVTKPKTAKPKKAAPKKK
KPKKVAK**A**PAKAKAVKPKAAKARVTKPKTAKPKKAAPKKK

Protein sequences of human histone H1.1 constructs: a, full-length; b, C-terminal deletion mutants; c, N-terminal deletion mutants; d, substitution of Threonine 152 to alanine (full-length) and e, substitution of Serine 183 to alanine (full-length). The underlined region represents the central globular domain of H1.1 and the bolded "A" represents the substituted alanine residues.